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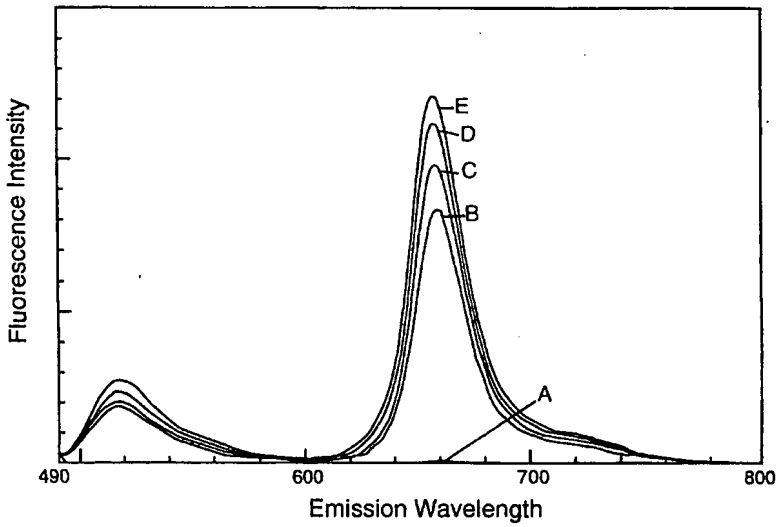

(57) Abstract

Energy transfer compositions comprising one or more fluorescent dyes and a fluorescent protein are described, in particular where the fluorescent dye is a sulfonated dye and the fluorescent protein is a phycobiliprotein. The energy transfer compositions of the invention may further comprise additional fluorescent dyes or fluorescent proteins that act as intermediate energy transfer dyes or ultimate emitter dyes. The energy transfer compositions of the invention may also be substituted by chemically reactive functional groups, or covalently bound conjugated substances. The compositions of the invention possess utility as detection reagents and as fluorescent tracers in a wide variety of applications, including biological applications.

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ENERGY TRANSFER COMPOSITIONS COMPRISING PHYCOBILIPROTEINS

FIELD OF THE INVENTION

The invention relates to fluorescent energy transfer compositions comprising one or more fluorescent dyes and a fluorescent protein, particularly a phycobiliprotein.

BACKGROUND

Fluorescence Resonance energy transfer (FRET) is a process whereby a first fluorescent dye (the "donor" dye) is excited, typically by illumination, and transfers its absorbed energy to a second fluorescent dye (the "acceptor" dye) that has a longer wavelength and therefore lower energy emission. The efficiency of the energy transfer is governed by several factors, including the distance between the donor dye and the acceptor dye, and the degree of spectral overlap between the emission wavelengths of the donor dye and the absorption wavelengths of the acceptor dye. The difference between the excitation wavelength and the emission wavelength of a fluorescent dye is called the "Stokes shift" of that dye.

Some artificial fluorescent labels utilize FRET to produce large effective Stokes shifts, the magnitude of which is rarely seen in a single fluorescent dye. Pairs of dissimilar dyes tethered by a covalent linkage such that they undergo energy transfer typically possess utility as labels for specific binding pair members, such as antibodies and oligonucleotides. Such "bifluorophore" labels permit the use of a single excitation source (frequently a laser) to simultaneously excite a variety of fluorescent labels, that may then be detected at widely separated wavelengths.

Single phycobiliproteins have been used as fluorescent labels for a variety of biomolecules. In addition, useful fluorescent labels have been prepared that utilize energy transfer pairs incorporating two phycobiliproteins or a phycobiliprotein and an organic fluorescent dye.

Where a synthetic dye is used as the ultimate emitter in an energy transfer pair, the emission intensity of the conjugate is typically less intense than could be obtained using a phycobiliprotein as the ultimate emitter and taking advantage of the exceptionally high absorbance and high fluorescence quantum yields of phycobiliproteins.

The present invention describes energy transfer compositions comprising a synthetic dye and a fluorescent protein, where the fluorescent protein acts as an energy acceptor.

The compositions of the present invention are brightly fluorescent, undergo efficient energy transfer, and have exceptionally large effective Stokes shifts.

In one aspect of the invention. the subject energy transfer compositions comprise an intrinsically fluorescent protein that has been chemically crosslinked acting as an acceptor for a synthetic donor dye.

In another aspect of the invention, the energy transfer compositions comprise an intrinsically fluorescent protein that acts as an acceptor for a synthetic donor dye, where the donor dye is sulfonated one or more times.

In another aspect of the invention, the energy transfer compositions comprise an intrinsically fluorescent protein that acts as an acceptor from a synthetic donor dye, where the compositions exhibit an effective Stokes shift of greater than 100 nm.

In another aspect of the invention, the energy transfer compositions comprise an intrinsically fluorescent protein that acts as an acceptor from a synthetic donor dye, where the compositions further comprise one or more additional synthetic dyes that act as intermediate energy transfer dyes.

In another aspect of the invention, the energy transfer compositions comprise an intrinsically fluorescent protein that acts as an acceptor from a synthetic donor dye, where the compositions further comprise one or more additional synthetic dyes or fluorescent proteins that act as final emitter dyes (ultimate acceptors), yielding even greater effective Stokes shifts.

In another aspect of the invention, the energy transfer compositions comprise an intrinsically fluorescent protein that acts as an acceptor from a synthetic donor dye, where the compositions are further substituted by chemically reactive functional groups, or by covalently bound conjugated substances.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: The fluorescence emission spectra of an energy transfer composition utilizing ALEXA FLUOR 488 dye and crosslinked allophycocyanin (XL-APC). Fluorescence emission is plotted as a function of the number of ALEXA FLUOR 488 fluorophores on the protein (degree of substitution, or DOS). A) XL-APC only; B) DOS = 12.7; C) DOS = 15; D) DOS = 26; E) DOS = 33.

Figure 2: The fluorescence emission spectra of an energy transfer composition utilizing fluorescein isothiocyanate (FITC) dye and XL-APC. Fluorescence emission is plotted as a

function of the fluorescein DOS. A) XL-APC only; B) DOS = 11.5; C) DOS = 15.5; D) DOS = 23.

Figure 3. The fluorescence emission spectra of an energy transfer composition utilizing OREGON GREEN 488 dye and XL-APC. Fluorescence emission is plotted as a function of the fluorophore DOS. A) XL-APC only; B) DOS = 16.4; C) DOS = 20.

Figure 4. The fluorescence emission spectra of an energy transfer composition utilizing CY3 dye and XL-APC. Fluorescence emission is plotted as a function of fluorophore DOS. A) XL-APC only; B) DOS = 2.4; C) DOS = 4.2; D) DOS = 5.2.

Figure 5. The fluorescence emission spectra of an energy transfer composition utilizing OREGON GREEN 488 dye and R-phycoerythrin (R-PE). Fluorescence emission is plotted as a function of fluorophore DOS. A) R-PE only; B) DOS = 6.5; C) DOS = 10.9; D) DOS = 12.9.

Figure 6. The fluorescence emission spectra of an energy transfer composition utilizing ALEXA FLUOR 350 dye and R-phycoerythrin (R-PE). Fluorescence emission is plotted as a function of fluorophore DOS. A) R-PE only; B) DOS = 7.4; C) DOS = 11.7; D) DOS = 16.2; E) DOS = 19.4; F) DOS = 23.8; G) DOS = 27.6.

Figure 7. A) The fluorescence emission spectrum of XL-APC when excited at 488 nm. B) The fluorescence emission spectrum of an energy transfer composition utilizing ALEXA FLUOR 488 dye and XL-APC (DOS = 16), when excited at 488 nm. B) The fluorescence emission spectrum of an energy transfer composition utilizing ALEXA FLUOR 488 dye (DOS = 16), XL-APC, and ALEXA FLUOR 568 dye (DOS = 2.0) when excited at 488 nm.

Figure 8. The fluorescence emission spectra of an energy transfer composition utilizing CASCADE BLUE dye and R-phycoerythrin (R-PE). Fluorescence emission is plotted as a function of fluorophore DOS. A) R-PE only; B) DOS = 4.2; C) DOS = 5.8; D) DOS = 7.6.

Figure 9. A comparison of the fluorescence emission spectra of A) an energy transfer composition utilizing R-phycoerythrin (R-PE) and TEXAS RED dye, and B) an energy transfer composition utilizing CASCADE BLUE dye, R-phycoerythrin (R-PE), and TEXAS RED dye. Both compositions were excited at 375 nm. The spectra show improved emission

from the TEXAS RED dye when a fluorophore with improved absorbance at shorter wavelengths is also conjugated to the phycobiliprotein.

Figure 10. A comparison of the fluorescence emission spectra of A) ALEXA FLUOR 488 dye, and B) The ALEXA FLUOR 488 dye–XL-APC composition shows that extensive energy transfer occurs between the ALEXA FLUOR 488 dye and the phycobiliprotein (excitation at 485 nm).

Figure 11. The excitation spectra of A) XL-APC alone and B) the ALEXA FLUOR 488 dye–XL-APC composition, showing the enhanced excitation of the energy transfer composition in the 480-510 nm range, relative to the phycobiliprotein alone.

Figure 12. A comparison of A) the fluorescence emission spectrum of XL-APC, when excited at 640 nm, and B) the fluorescence emission spectrum of ALEXA FLUOR 488 dye–XL-APC, when excited at 640 nm (as described in Example 6). The spectrum of the energy transfer composition shows little effect on the quantum yield of the phycobiliprotein due to its chemical modification.

Figure 13. The effect of degree of substitution on FRET efficiency. Increasing dye:protein molar ratios during the preparation of the energy transfer compositions of the invention yields increasing FRET efficiency. A) Fluorescence emission spectra of ALEXA FLUOR 488 dye–XL-APC prepared at a dye:protein ratio of 35; B) Fluorescence emission spectra of ALEXA FLUOR 488 dye–XL-APC prepared at a dye:protein ratio of 45; C) Fluorescence emission spectra of ALEXA FLUOR 488 dye–XL-APC prepared at a dye:protein ratio of 60 (Example 7)

Figure 14. The effect of salt and chemical cross-linking on phycobiliprotein stability. A comparison of the fluorescence emission spectra of A) carboxyfluorescein and XL-APC at a molar ratio of 25; B) carboxyfluorescein and XL-APC at a molar ratio of 25 in the presence of 10% sodium sulfate; C) carboxyfluorescein and native APC at a molar ratio of 25; D) carboxyfluorescein and native APC at a molar ratio of 25 in the presence of 10% sodium sulfate (Example 8).

Figure 15. A comparison of the absorption spectra of the energy transfer compositions A) ALEXA FLUOR 488 dye–APC; and B) ALEXA FLUOR 488 dye–XL-APC (Example 9).

Figure 16. A comparison of flow cytometric analysis of Jurkat cells with streptavidin-modified ALEXA FLUOR 488 dye–XL-APC and with streptavidin-RED 670 (Example 13).

Figure 17. Flow cytometric analysis of Jurkat cells using three-color analysis: SYTOX GREEN nucleic acid stain (green fluorescent) for evaluation of cell viability, R-PE labeled anti-CD4 (orange fluorescent) and streptavidin-modified ALEXA FLUOR 488 dye–XL-APC in combination with biotinylated anti-CD3 (red to infrared fluorescent). Figure 17A shows the total population of cells under analysis, based on light scattering properties. Figure 17B shows the portion of the cells analyzed that were not stained by any of the three fluorescent probes. Figure 17C shows the cell population that exhibited low green fluorescence (or viable cells). Figure 17D shows the cell population that was stained by all three fluorescent probes.

SUMMARY OF THE INVENTION

The present invention describes novel energy transfer compositions comprising a synthetic dye and a fluorescent protein. The synthetic dye is optionally a sulfonated dye. The fluorescent protein is optionally a phycobiliprotein. In one embodiment the phycobiliprotein subunits have been chemically cross-linked. The energy transfer compositions of the invention optionally further comprise additional synthetic dyes or fluorescent proteins that act as intermediate energy transfer dyes or ultimate emitter dyes. The energy transfer compositions of the invention are optionally substituted by chemically reactive functional groups, or by covalently bound conjugated substances.

The energy transfer compositions of the invention typically have the formula

$$(D)_p(A)$$

wherein A is an intrinsically fluorescent protein and D is a fluorescent moiety having a molecular weight less than 2,000 that is covalently bound to A. Typically D has a fluorescence emission maximum at a shorter wavelength than the fluorescence emission maximum of A, and acts as an energy donor, while A acts as an energy acceptor. The integer p (or DOS) is the number of molecules of the fluorescent moiety D that are covalently attached to one molecule of the fluorescent protein A, where p is typically an integer from 1 to 30.

The Fluorescent Protein A

By "fluorescent protein" is meant a protein that has intrinsic fluorescence in the visible or infrared region of the electromagnetic spectrum, with a fluorescence emission maximum at a wavelength beyond about 380 nm. Preferred fluorescent proteins are those of abundant natural origin, such as from algae or an animal, or their genetically modified forms, and typically has a molecular weight less than 500,000, more typically less than 300,000. Typically the fluorescent proteins are easily purified, reasonably stable in solution and have high molar absorptivity. Typically the fluorescent proteins exhibit extinction coefficients greater than 10^5 $cm^{-1}M^{-1}$, often greater than 10^6 $cm^{-1}M^{-1}$, and fluorescence quantum yields greater than 0.5. The fluorescent proteins typically exhibit a fluorescence emission maximum ($\lambda_{Em\text{-}max}$) at a wavelength beyond 450 nm, preferably beyond about 560 nm, and in some cases beyond about 650 nm.

Preferably the fluorescent proteins of the invention are phycobiliproteins, such as phycoerythrins, phycocyanins or allophycocyanins. Most preferred are B- or R-phycoerythrins for maximum emission wavelengths at less than about 600 nm and allophycocyanins (APC) for maximum emission wavelengths of greater than about 650 nm. Other intrinsically fluorescent proteins that emit maximally beyond 400 nm include peridinin chlorophyll protein (PerCp) and natural and genetically modified green fluorescent protein (GFP).

In one embodiment, subunits of the fluorescent protein of the invention are chemically cross-linked (Examples 1, 2, and 3 and Table 2). Chemical crosslinking of protein subunits of phycobiliproteins significantly improves the fluorescence of the compositions, particularly in the case of allophycocyanin. Energy transfer compositions that incorporate a crosslinked protein exhibit enhanced fluorescence, relative to compositions that incorporate the native protein. Cross-linking also helps prevent dissociation of the phycobiliprotein upon conjugation to a high number of dyes.

Typically, the energy transfer compositions of the invention comprise multiple identical donor dyes attached to a single fluorescent protein. Preferably, the fluorescent protein A is covalently attached to 1 - 30 identical fluorescent moieties that function as donor dyes for energy transfer to the fluorescent protein. The optimal DOS of a given donor dye on a particular fluorescent protein is readily determined by experimentation, using methods well known in the art (Example 1, Figures 1-6).

In order to be readily and easily utilized with existing fluorescence instrumentation,

preferred fluorescent donor dyes are those having strong absorption bands at the output wavelengths of commonly utilized excitation sources. Preferred donor dyes are those that are readily excited at 350-365 nm, 400-410 nm, 442 nm, 488 nm, 514 nm, 532 nm, 540-550 nm, 568 nm, 590-600 nm, or 630-650 nm, and possess an extinction coefficient in at least one of these wavelength ranges that is greater than about 15,000 $cm^{-1}M^{-1}$, more preferably greater than about 30,000 $cm^{-1}M^{-1}$, even more preferably greater than about 60,000 $cm^{-1}M^{-1}$, and most preferably greater than about 80,000 $cm^{-1}M^{-1}$. In a particularly preferred embodiment, the donor dyes are those that are optimally excited at 480-520 nm.

Typically the fluorescent moiety D has a molecular weight of less than about 2,000, preferably less than 1,000. The fluorescence emission maximum ($\lambda_{Em\text{-}max}$) of the fluorescent moiety is typically at least 25 nm lower than the maximum absorbance wavelength ($\lambda_{Ab\text{-}max}$) of the fluorescent protein, more preferably more than 50 nm lower, and even more preferably more than 100, 140, or even 200 nm lower than that of the fluorescent protein.

Also preferred are donor dyes that possess intrinsic fluorescence quantum yields when covalently bound to a non-fluorescent protein (for example an immunoglobulin) of greater than about 0.1, more preferably greater than about 0.2, yet more preferably greater than about 0.4, and the most preferred dyes have a quantum yield on proteins of greater than about 0.6. Typically the preferred dyes of the invention do not exhibit significant fluorescence quenching even when 4 or more molecules of the dye, to as many as 10 dyes per molecule, are conjugated to an immunoglobulin having a molecular weight of greater than about 140,000.

Preferred donor dyes also exhibit relatively prompt emission, that is they have excited state lifetimes of less than about 100 nsec. The fluorescence of such donor dyes typically can be detected during the transit of the dye or dye-conjugate through a beam of exciting light. For this reason the use of rare earth dye complexes such as those of terbium and europium as donors is not preferred, and in some applications may not be suitable.

It is most advantageous if the donor dye-fluorescent protein composition exhibits efficient FRET, that is most of the light absorbed at the excitation wavelength of the donor dye results in emission at or near the emission maximum of the fluorescent protein. Although it is possible to detect and quantitate FRET emission that is much less than quantitative, for practical purposes it is preferred that the energy transfer of the instant compositions is greater than about 50% efficient, as measured by the decrease in donor dye fluorescence (Example 5). Other preferred energy transfer compositions exhibit transfer efficiencies of greater than about 60%, 70%, 80%, 90%, 95%, or most preferably greater than about 98% when measured against the free, unconjugated dye in the same medium at

the same absorbance intensity.

For preferred embodiments, little or no fluorescence emission corresponding to the normal emission maximum of the donor dye(s) should be detected (Example 5, Figure 10). Preferably, the intensity of the donor dye emission is less than half the fluorescence intensity of the long wavelength emission, more preferably less than 30%, 20%, 15%, 10%, 5%, or most preferably less than 2% the fluorescence intensity of the long wavelength emission peak. However, even where significant donor emission intensity remains, measurement and compensation means are well known that minimize interference from residual donor dye emission.

The most preferred energy transfer compositions exhibit a maximum fluorescence emission at greater than about 560 nm (more preferably greater than about 620 nm and yet more preferably greater than about 650 nm), exhibit a short wavelength donor dye emission that is less than about 20% of the intensity of the longer wavelength peak (more preferably less than about 10%, and yet more preferably less than about 5%, and most preferably less than about 2%).

Due to efficient energy transfer from the donor dye, the compositions of the invention typically exhibit greater fluorescence at the maximal emission wavelength of the fluorescent protein when excited at the excitation maximum of the donor dye than the fluorescent protein alone exhibits when excited at the same wavelength (See Figure 7). Although even small fluorescence enhancements may be useful, a relative fluorescence increase of at least 30% upon conjugation to the donor dye is typical, and enhancement of greater than about 50% is preferred. Similarly, a two-fold enhancement in relative fluorescence upon conjugation is useful, a five-fold enhancement is preferred, a ten-fold enhancement is more preferable, a twenty-fold enhancement is yet more preferably, and a forty-fold enhancement is exceptionally useful.

In another embodiment of the invention, the energy-transfer compositions of the invention incorporate one or more additional fluorescent moieties that exhibit maximal absorption and emission wavelengths that are intermediate between those of the first fluorescent donor dye and the fluorescent protein. These intermediate "transfer dyes" typically improve the efficiency of FRET between the initial donor dye and the acceptor fluorescent protein, and results in decreased residual fluorescence from the first donor dye (Example 2). In some cases, addition of the transfer dye also results in enhanced fluorescence emission from the protein acceptor.

As with the first fluorescent donor dyes, the intermediate transfer fluorophores are covalently bound to the fluorescent protein. The degree of substitution is readily optimized

by experimentation, using methods known in the art (Example 1). The energy transfer compositions of the invention typically comprise 1-10 distinct types of transfer dyes, there being from 1-30 individual dyes of each type bound to the fluorescent protein. Typically, 1-3 types of transfer dyes are used, and typically the degree of substitution of the transfer dye is less than that of the initial donor dye. As is readily understood, in order for the transfer dyes to be useful for energy transfer, they are each distinct from the initial donor dye, and typically have both a maximum absorption wavelength ($\lambda_{Ab\text{-}max}$) and a fluorescence emission maximum wavelength ($\lambda_{Ab\text{-}max}$) that lie between the maximum absorption wavelength of the donor dye and the fluorescence emission wavelength of the fluorescent protein. In a preferred embodiment, the additional fluorescent moiety is a fluorescein, a rhodol, a rhodamine, a cyanine, a polyazaindacene, or an oxazine dye.

In yet another embodiment of the invention, the energy transfer composition further comprises one or more additional dyes that have absorption maxima that overlap the emission of the fluorescent protein and exhibit maximal fluorescence emission at longer wavelengths than that of the fluorescent protein. That is, these additional dyes function as final acceptor dyes, receiving energy from the fluorescent protein and emitting fluorescence at an even longer wavelength. These compositions typically exhibit an exceptionally high shift in the emission relative to the exciting light. Although a second fluorescent protein may be used as an ultimate acceptor, the ultimate acceptor is preferably a synthetic dye of molecular weight less than about 2,000 that has maximal absorption that overlaps the emission of the protein dye. Preferably these dyes are cyanines, including sulfonated cyanines.

The energy transfer compositions of the invention optionally further comprise a chemically reactive functional group that is covalently attached to the fluorescent protein, in order to permit the covalent conjugation of the energy transfer composition to a surface, a carrier, or a targeting molecule (typically a specific binding pair member) or a tracer. The chemically reactive functional group is typically one that will react with an amino, a thiol, an aldehyde, a ketone, a hydrazine, or a hydroxylamine derivative.

The Fluorescent Moiety

The fluorescent moiety is typically a synthetic dye that acts as an energy donor. However, additional fluorescent moieties may be utilized as intermediate transfer dyes, or even as an ultimate emitter, receiving energy transfer from the fluorescent protein A. As used herein, 'synthetic dye' is used to refer to fluorescent moieties useful as donor dyes,

intermediate dyes or ultimate emitter dyes. One of ordinary skill in the art is capable of determining the utility of a given fluorescent moiety as a donor dye vs. an intermediate dye by examination of that moiety's maximal excitation and emission bands with respect to the desired fluorescent protein.

The most preferred candidates for the synthetic dyes of the invention possess extinction coefficients greater than about 60,000 $cm^{-1}M^{-1}$ in the wavelength range of 485 nm to 515 nm. Their conjugates with nonfluorescent immunoglobulins exhibit a quantum yield of greater than 0.2, preferably greater than 0.4, and they do not exhibit significant fluorescence quenching upon conjugation to immunoglobulins unless more than 6, preferably more than 10, molecules of the dye are conjugated to the same immunoglobulin.

A wide variety of chemically reactive fluorescent dyes that would be suitable for incorporation into the energy transfer compositions of the invention are already known in the art (see for example MOLECULAR PROBES HANDBOOK, Sixth Ed., Richard P. Haugland, ed. (1996), in particular Chapters 1-3; BIOPROBES 26 (October 1997); BIOPROBES 27 (February 1998); BIOPROBES 28 (May 1998); and BIOPROBES 29 (November 1998)). The spectral properties of candidate dyes in solution or when conjugated to proteins such as IgG are known or are readily measured using a spectrofluorometer or spectrophotometer.

Typically, the synthetic dye (whether a donor dye, intermediate transfer dye, or ultimate emitter dye) is a pyrene, an anthracene, a naphthalene, an acridine, a stilbene, an indole or benzindole, an oxazole or benzoxazole, a thiazole or benzothiazole, a 4-amino-7-nitrobenz-2-oxa-1,3-diazole (NBD), a cyanine, a carbocyanine, a carbostyryl, a porphyrin, a salicylate, an anthranilate, an azulene, a perylene, a pyridine, a quinoline, a coumarin (including hydroxycoumarins and aminocoumarins and fluorinated derivatives thereof (as described in U.S. Patent No. 5,830,912 to Gee et al. (1998)), a 4-bora-3a,4a-diaza-s-indacene (e.g. US Patents 4,774,339 to Haugland, et al. (1988); 5,187,288 to Kang, et al. (1993); 5,248,782 to Haugland, et al. (1993); 5,274,113 to Kang, et al. (1993); and 5,433,896 to Kang, et al.(1995)), a xanthene, an oxazine or a benzoxazine, a carbazine (US Patent 4,810,636 to Corey (1989)), or a phenalenone or benzphenalenone (US Patent 4,812,409 Babb et al. (1989)). Preferably, the donor dye is a carbazine, an oxazine, a coumarin, a pyrene, a xanthene, a naphthalene, a phenalenone, or a 4-bora-3a,4a-diaza-s-indacene.

Where the synthetic dye is a xanthene, the synthetic dye is optionally a fluorescein, a rhodol (US Patent 5,227,487 to Haugland, et al. (1993)), or a rhodamine. As used herein, fluorescein includes benzo- or dibenzofluoresceins, seminaphthofluoresceins, or naphthofluoresceins. Similarly, as used herein rhodol includes seminaphthorhodafluors

(US Patent 4,945,171 to Haugland. et al. (1990)). Fluorinated xanthene dyes have also been described previously (Int. Publ. No. WO 97/39064, Molecular Probes, Inc. (1997)). As used herein, oxazines include resorufins, aminooxazinones, diaminooxazines, and their benzo-substituted analogs.

In one embodiment, the synthetic dye is a sulfonated dye. The sulfonation of fluorescent dyes (the substitution of a sulfonic acid moiety or a salt of a sulfonic acid) typically results in enhanced dye solubility in aqueous solutions. In addition, sulfonation of some cyanine dyes has been shown to decrease the inherent tendency of those dyes to form dimers and aggregates, presumably due to the increased polar character imparted by the sulfonic acid moiety. Ring sulfonation may enhance dye photostability (resistance to degradation upon illumination), and may decrease fluorescence quenching upon conjugation to biomolecules. We have found that sulfonated dyes consistently give higher fluorescence yields, more efficient energy transfer and are more resistant to precipitation or other artifacts than nonsulfonated dyes that have similar spectral properties. In preferred embodiments, the sulfonated dye of the invention is a sulfonated pyrene, coumarin, cyanine, or xanthene (including sulfonated fluoresceins, rhodols and rhodamines).

Preferably the sulfonated synthetic dye is a pyrene, a coumarin, a carbocyanine, or a xanthene dye that has been sulfonated one or more times (as described in U.S. Patent No. 5,132,432 to Haugland et al., (1992); U.S. Patent No. 5,696,157 to Wang et al. (1997); U.S. Patent No. 5,268,486 patent to Waggoner et al. (1993); and Int. Publ. No. WO 99/15517 (PCT/US98/19921)). Sulfonated pyrenes and coumarins are typically excited at wavelengths below about 450 nm. Sulfonated xanthenes, and in particular sulfonated rhodamine dyes, are particularly preferred as the fluorescent donor dyes of the invention. Sulfonated dyes that absorb maximally beyond about 515 nm, such as sulfonated rhodamines, are also preferred, as are sulfonated cyanines.

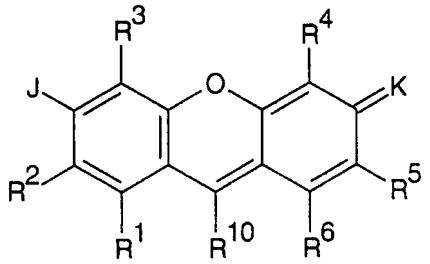
Sulfonated Xanthene Dyes

Preferably, at least one synthetic dye of the invention is a sulfonated xanthene. Sulfonated xanthene, as used herein, includes fluoresceins, rhodamines and rhodols that are substituted one or more times by $-SO_3X$ or $-CH_2SO_3X$, where X is H (sulfonic acid), or a counterion (salt of a sulfonic acid). As used herein, where X is a counterion, it is typically a cation that is not toxic as used, and does not have a substantially deleterious effect on biomolecules. Examples of suitable cations include without limitation K^+, Na^+, Cs^+, Li^+, Ca^{2+}, Mg^{2+}, ammonium, alkylammonium or alkoxyammonium salts, or pyridinium salts.

Alternatively, the counterion of the sulfonic acid may form an inner salt with a positively charged atom on the xanthene dye itself, typically the quaternary nitrogen atom of a rhodamine dye.

As discussed above, each synthetic dye is attached to the fluorescent protein (A) via a covalent linkage, L. In the description of the sulfonated xanthene dyes below, the covalently bound fluorescent protein is represented herein as an -L-A moiety. The covalent linkage L binds the sulfonated xanthene to the fluorescent protein either directly (L is a single bond) or with a combination of stable chemical bonds, optionally including single, double, triple or aromatic carbon-carbon bonds, as well as carbon-nitrogen bonds, nitrogen-nitrogen bonds, carbon-oxygen bonds, carbon-sulfur bonds, phosphorus-oxygen bonds, and phosphorus-nitrogen bonds. L typically includes ether, thioether, carboxamide, sulfonamide, urea, urethane or hydrazine moieties. Preferred L moieties have 1-20 nonhydrogen atoms selected from the group consisting of C, N, O, P, and S; and are composed of any combination of ether, thioether, amine, ester, carboxamide, sulfonamide, hydrazide bonds and aromatic or heteroaromatic bonds. Preferably L is or incorporates a carboxamide, sulfonamide or thiourea moiety.

In one embodiment, the sulfonated xanthene dyes have the formula



Substituents R^2, R^3, R^4 and R^5 are independently H, F, Cl, Br, I, CN; or C_1-C_{18} alkyl, or C_1-C_{18} alkoxy, where each alkyl or alkoxy is optionally further substituted by F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C_1-C_6 alcohol. Alternatively, one of R^2, R^3, R^4 and R^5 is -L-A). In a preferred embodiment, R^3 and R^4 are each $-SO_3X$.

Substituents R^1 and R^6 are H, or R^1 taken in combination with R^2, or R^5 taken in combination with R^6, or both, form a fused aromatic six membered ring, that is optionally substituted by one or more $-SO_3X$ moieties.

In one embodiment of the invention, R^2, R^3, R^4 and R^5 are independently H, F, Cl,

Br, I or C_1-C_{18} alkyl. In another embodiment of the invention, R^1, R^2, R^5 and R^6 are H. In yet another embodiment of the invention, R^2 and R^5 are each F or Cl.

The J moiety is OR^7, where R^7 is H, C_1-C_{18} alkyl, or is -L-A. Alternatively, J is NR^8R^9 where R^8 and R^9 are independently H, C_1-C_6 alkyl, C_1-C_6 carboxyalkyl, C_1-C_6 sulfoalkyl, a salt of C_1-C_6 carboxyalkyl, or a salt of C_1-C_6 sulfoalkyl, where the alkyl portions each are independently and optionally substituted by amino, hydroxy, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C_1-C_6 alkyl. Alternatively, R^8 in combination with R^9 forms a saturated 5- or 6-membered heterocycle that is a piperidine, a morpholine, a pyrrolidine or a piperazine, each of which is optionally substituted by methyl, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C_1-C_6 alkyl. In another alternative, one of R^8 and R^9 is -L-A.

In another aspect of the invention, R^8 in combination with R^2, or R^9 in combination with R^3, or both, form a 5- or 6-membered ring that is saturated or unsaturated, and is optionally substituted by one or more C_1-C_6 alkyls or $-CH_2SO_3X$ moieties.

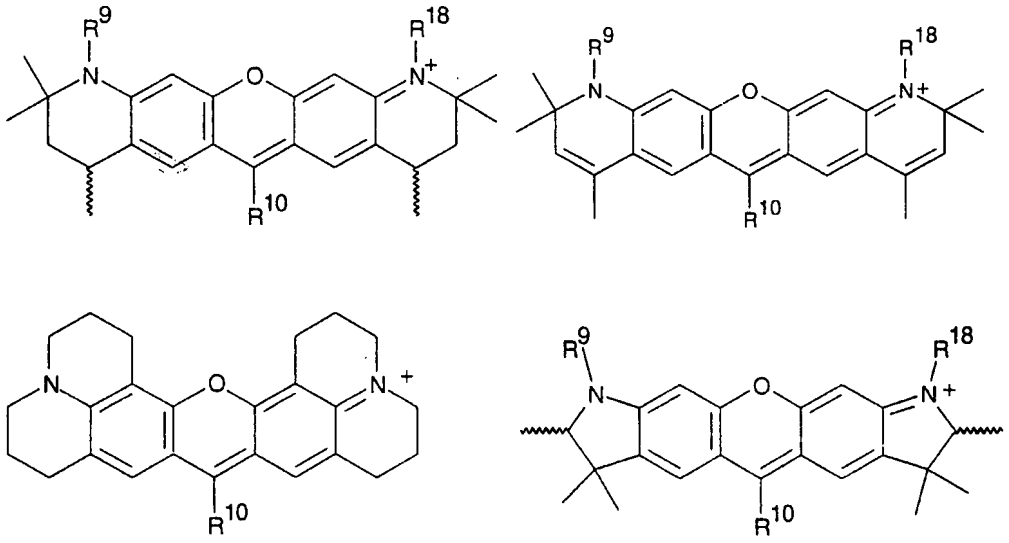
The K moiety is O or $N^+R^{18}R^{19}$, where R^{18} and R^{19} are independently H, C_1-C_6 alkyl, C_1-C_6 carboxyalkyl, C_1-C_6 sulfoalkyl, a salt of C_1-C_6 carboxyalkyl, or a salt of C_1-C_6 sulfoalkyl, wherein the alkyl portions are optionally substituted by amino, hydroxy, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C_1-C_6 alkyl. Alternatively, R^{18} in combination with R^{19} forms a saturated 5- or 6-membered heterocycle that is a piperidine, a morpholine, a pyrrolidine or a piperazine, each of which is optionally substituted by methyl, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C_1-C_6 alkyl. In another alternative, one of R^{18} and R^{19} is -L-A.

In another aspect of the invention, R^{18} in combination with R^4, or R^{19} in combination with R^5, or both, form a 5- or 6-membered ring that is saturated or unsaturated, and is optionally substituted by one or more C_1-C_6 alkyls or $-CH_2SO_3X$ moieties.

In one embodiment of the invention, R^9 and R^{18} are independently H, or carboxyalkyl, salt of carboxyalkyl, sulfoalkyl or a salt of sulfoalkyl, each having 1-6 carbons. Typically R^9 and R^{18} are H, methyl or ethyl.

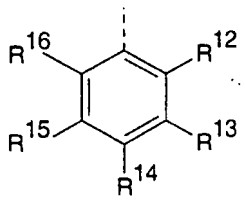
In another embodiment of the invention, R^8 in combination with R^2 and R^{19} in combination with R^5 independently form 5- or 6-membered rings that are saturated or unsaturated, and are optionally substituted by one or more alkyl groups having 1-6 carbons, or by one or more $-CH_2SO_3X$ moieties. In yet another embodiment of the invention, R^8 in combination with R^2 and R^{19} in combination with R^5 independently form 5- or 6-membered rings that are saturated, and are substituted by one or more $-CH_2SO_3X$ moieties. Some (but not all) examples of fused 5- or 6-membered rings as described herein

are provided below (additional substituents, such as sulfonic acid or sulfomethyl moieties not shown).



The substituent R^{10} is H, F, CN, a carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C_1-C_6 alcohol. Alternatively R^{10} is a saturated or unsaturated C_1-C_{18} alkyl that is optionally substituted one or more times by F, Cl, Br, carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of a C_1-C_6 alcohol, $-SO_3X$, amino, alkylamino, or dialkylamino, the alkyl groups of each substituent having 1-6 carbons. R^{10} is optionally -L-A.

In another embodiment of the invention, R^{10} is an aryl substituent having the formula



where the R^{12}, R^{13}, R^{14}, R^{15} and R^{16} substituents are independently H, F, Cl, Br, I, $-SO_3X$, a carboxylic acid, a salt of carboxylic acid, CN, hydroxy, amino, or hydrazino. Alternatively, one pair of adjacent substituents R^{13} and R^{14}, R^{14} and R^{15} or R^{15} and R^{16}, when taken in

combination, form a fused 6-membered aromatic ring that is optionally further substituted by carboxylic acid, or a salt of carboxylic acid. Alternatively, one of R^{12}, R^{13}, R^{14}, R^{15} and R^{16} is -L-A.

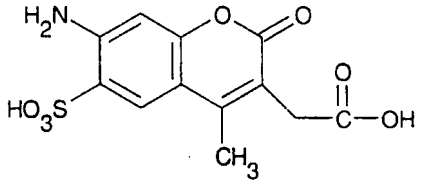
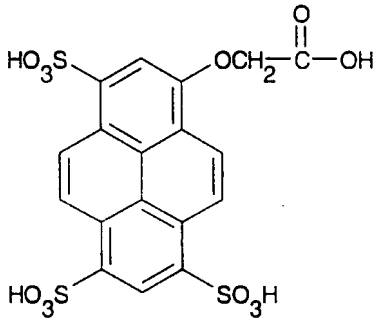
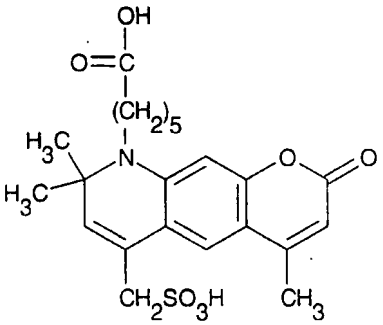
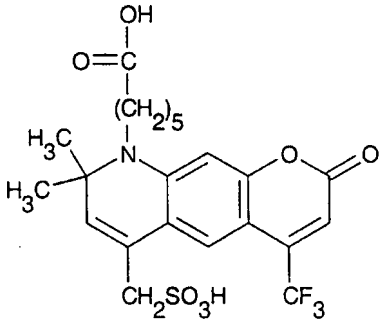
In one embodiment of the invention, R^{12}, R^{13}, R^{14}, R^{15}, and R^{16} are independently H, Cl, F, amino, nitro, $-SO_3X$, a carboxylic acid, a salt of carboxylic acid, or a carboxy-substituted alkylthio having the formula $-S-(CH_2)_nCOOH$, where n is 1-15. In another embodiment of the invention, at least three of R^{13}, R^{14}, R^{15}, and R^{16} are F or Cl. In another embodiment of the invention, one of R^{14} and R^{15} is a carboxylic acid, a salt of a carboxylic acid, or $-S-(CH_2)_nCOOH$, where n is 1-15, and the other of R^{14} and R^{15} is H, F or Cl.

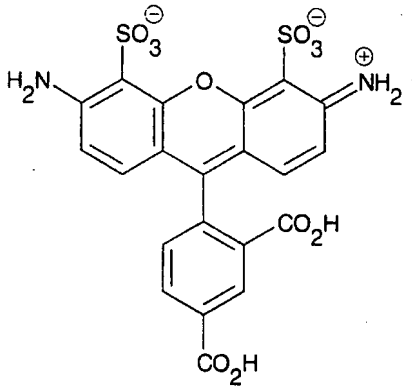
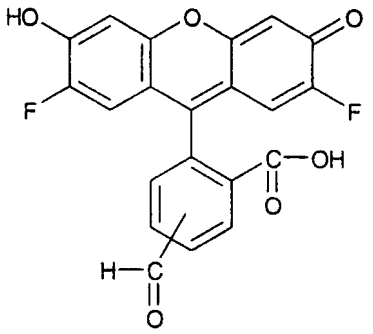
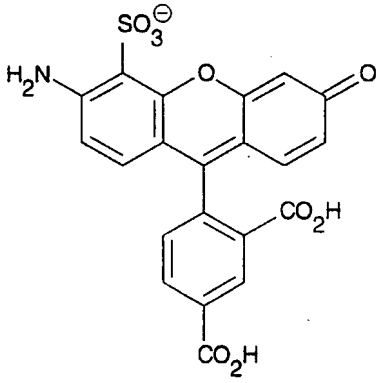
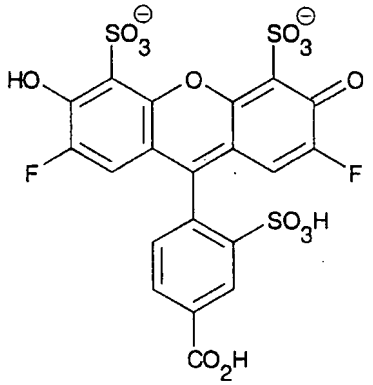
Where J is OR^7, K is O, R^{10} is aryl and R^{12} is carboxy or $-SO_3X$, the described dye is a fluorescein. Where J is NR^8R^9, K is O, R^{10} is aryl and R^{12} is carboxy, the described dye is a rhodol. Where J is NR^8R^9, K is $N^+R^{18}R^{19}$, R^{10} is aryl, and R^{12} is carboxy, the described dye is a rhodamine. Where the dyes of the invention are fluoresceins, they are preferably sulfonefluoresceins (wherein R^{12} is $-SO_3X$). Preferably, the dyes of the invention are rhodamines or rhodols, more preferably rhodamines.

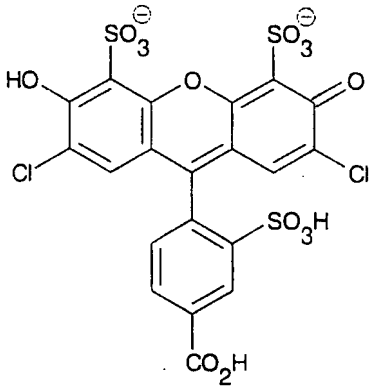
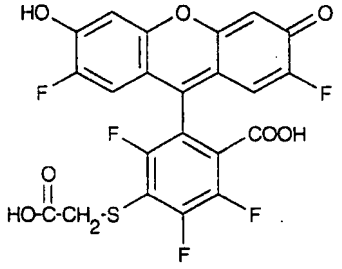
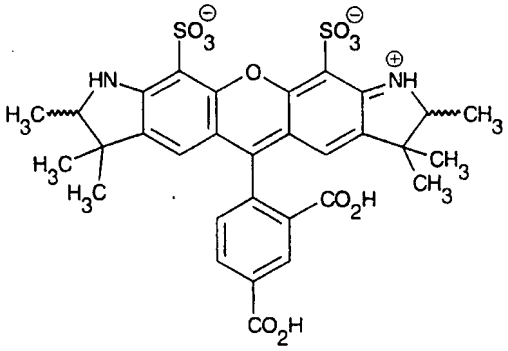
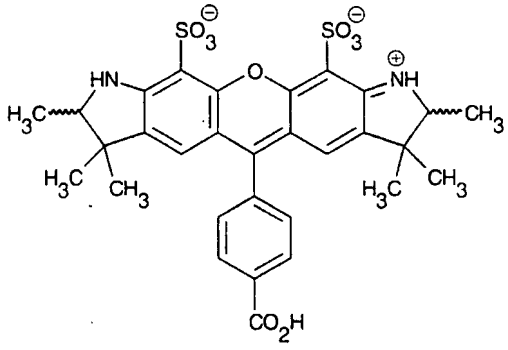
In one embodiment of the invention, at least one of R^2, R^3, R^4, and R^5 is $-SO_3X$, preferably R^3 and R^4 are $-SO_3X$. In another embodiment of the invention, R^1 taken in combination with R^2, or R^5 taken in combination with R^6,or both, form a fused aromatic six-membered ring that is substituted by at least one $-SO_3X$ moiety. In another embodiment of the invention R^8 in combination with R^2, or R^9 in combination with R^3, or R^{18} in combination with R^4, or R^{19} in combination with R^5, form a 5- or 6-membered ring that is saturated or unsaturated, and is substituted by at least one $-CH_2SO_3X$ moiety. Preferably R^8 in combination with R^2 and R^{19} in combination with R^5, form a 5- or 6-membered ring that is saturated or unsaturated, and is substituted by at least one $-CH_2SO_3X$ moiety.

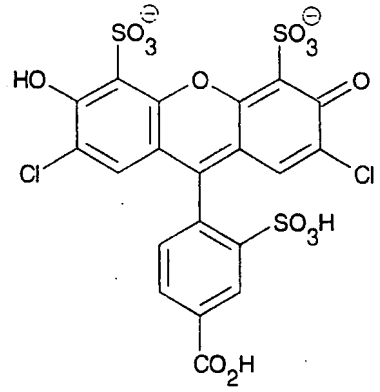
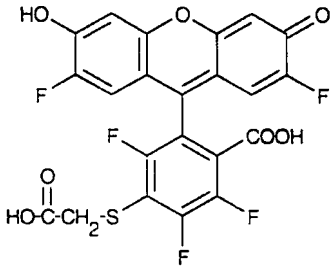
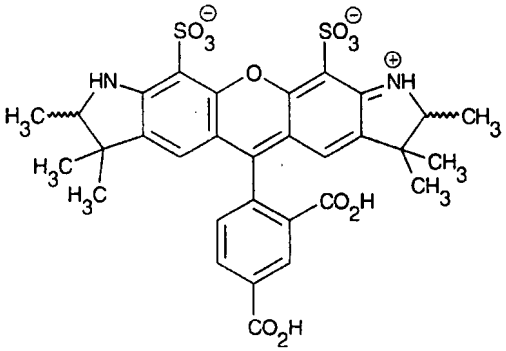
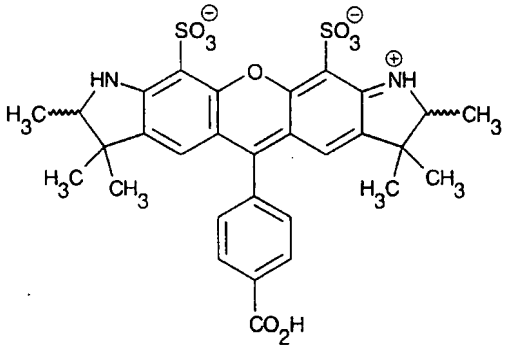
Spectral properties of some selected dyes are given in Table 1. Spectral properties of selected energy transfer compositions of the invention are given in Table 2.

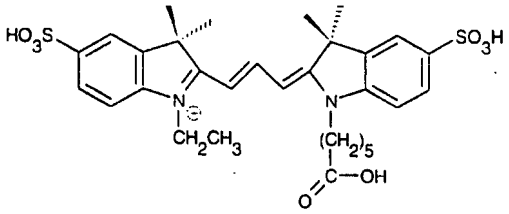
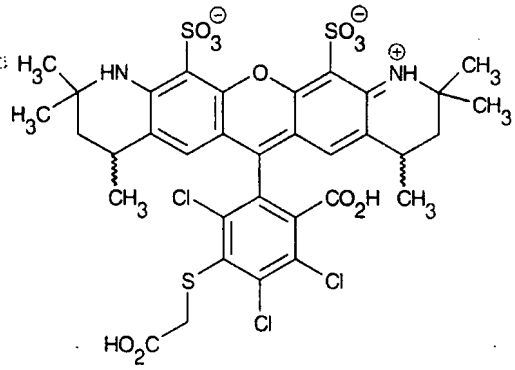
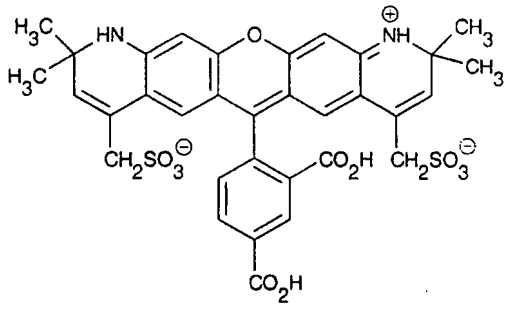
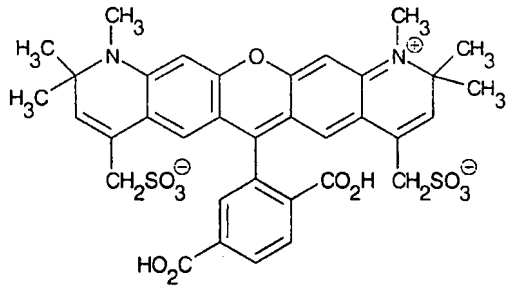
Table 1: Spectral properties of selected fluorophores useful for the preparation of energy transfer compositions of the invention.

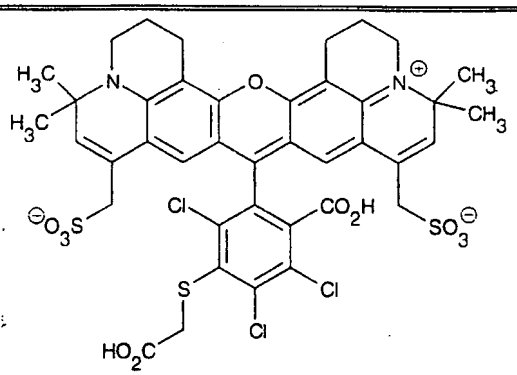
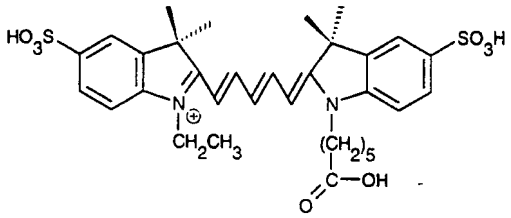
Dye No. (Trademark)†	Fluorophore‡	$\lambda_{Ab\text{-}max}$ (nm)	$\lambda_{Em\text{-}max}$ (nm)
1 (ALEXA FLUOR 350)		350	434
2 (CASCADE BLUE)		400	420
3		400	485
4 (ALEXA FLUOR 430)		430	545

5 (ALEXA FLUOR 488)		491	515
6 (OREGON GREEN 488)		492	516
7		493	518
8		496	514

9		506	522
10 (OREGON GREEN 514)		506	526
11		520	545
12 (ALEXA FLUOR 532)		523	548

9		506	522
10 (OREGON GREEN 514)		506	526
11		520	545
12 (ALEXA FLUOR 532)		523	548

13 (CY3)		550	570
14 (ALEXA FLUOR 546)		553	569
15 (ALEXA FLUOR 568)		573	596
16 (ALEXA FLUOR 594)		585	610

17		615	632
18 (CY5)		650	670

† Where the dye is commercially available under a trademark, the trademark is given.

‡ Although the carboxylic acid derivative of each fluorophore is shown, it should be recognized that a variety of other chemically reactive derivatives are readily prepared that are also useful for the preparation of the energy-transfer compositions of the invention (succinimidyl esters, maleimidyl derivatives, hydrazines, etc.).

Table 2. Selected Energy Transfer Compositions of the present invention.

Fluorophore	Phyco-biliprotein	Degree of Substitution	Excitation Wavelength (nm)	Fl. Int. at $Em_{max.}$ of Acceptor	Fl. Int. at Em_{max} of Donor	Increase in Fl. Int. at Em_{max} of Acceptor (1)	ET Efficiency (2)
ALEXA FLUOR 488	XL-APC	15.6	488	4.76	0.28	119	96%
ALEXA FLUOR 532	XL-APC	11.5	520	3.08	0.47	18.1	94%
ALEXA FLUOR 546	XL-APC	21.7	550	3.57	0.25	8.9	98%
ALEXA FLUOR 568	XL-APC	2.2	550	1.17	0.12	1.70	97%
ALEXA FLUOR 594	XL-APC	9.1	560	3.15	0.07	1.93	99%
BODIPY Fl	XL-APC	12.2	488	2.57	0.79	23	87%
Carboxy-fluorescein	XL-APC	13.9	488	3.90	0.41	72	82%
FITC	XL-APC	23.0	488	3.60	0.96	67	94%
OREGON GREEN 488	XL-APC	16.4	488	3.24	0.90	54	94%
CY3	XL-APC	5.2	550	2.85	0.10	7.1	92%
LASERPRO 790	XL-APC	6.9	635	0.90	1.63	4.0	82%
ALEXA FLUOR 350	R-PE	16.2	350	4.95	0.12	2.5	96%
CASCADE BLUDE	R-PE	7.2	375	5.02	0.08	2.0	99%
ALEXA FLUOR 488	R-PE	15.0	488	1.07	0.08	1.7	92%
FITC	R-PE	13.8	488	8.50	0.06	1.3	95%
OREGON GREEN 488	R-PE	6.5	488	0.80	0.01	1.3	97%
ALEXA FLUOR 488	B-PE	20.3	488	3.27	0.05	2.1	83%
FITC	B-PE	20.1	488	4.54	0.04	1.7	94%
OG 488	B-PE	27.0	488	2.73	0.05	1.8	90%

(1) Increase in fluorescence intensity of the acceptor dye in the conjugate relative to acceptor dye in solution. Same optical density at acceptor absorbance max.

(2) Decrease in fluorescence intensity of the donor dye in the conjugate relative to the donor dye in solution. Same optical density at excitation wavelength.

Chemically Reactive Compositions and Their Conjugates

Where the energy transfer composition contains a chemically reactive group (R_x), it is typically attached to the fluorescent protein by a covalent linkage L, as defined above. Each covalent linkage L present in the energy transfer composition is optionally the same or different. Typically, the conjugation reaction between the chemically reactive fluorescent protein and a substance to be conjugated results in one or more atoms of the reactive group R_x to be incorporated into a new linkage L attaching the energy transfer composition to the conjugated substance S_c. The covalent linkage L binds the reactive group R_x or conjugated substance S_c to the fluorescent protein, either directly (L is a single bond) or with a combination of stable chemical bonds, as described above.

Choice of the reactive group used to attach the energy transfer composition to the substance to be conjugated typically depends on the functional group on the substance to be conjugated and the type or length of covalent linkage desired. While a variety of reactive functional groups are known in the art, typically R_x is an acrylamide, an activated ester of a carboxylic acid, hydroxy, an aldehyde, an alkyl halide, a sulfonate ester, an amine, an anhydride, an aniline, an aryl halide, an azide, an aziridine, a boronate, a carboxylic acid, an epoxide, a glycol, a haloacetamide, a halotriazine, a hydrazine, a hydroxylamine, an isothiocyanate, a ketone, a maleimide, a thiol, or a disulfide group. More preferably, R_x is an activated ester of a carboxylic acid, a maleimide, a thiol, or a disulfide group.

Where the energy transfer compositions of the invention are substituted by R_x, the reactive functional group is typically present as 1-5 functional groups having the same chemical structure, each of which is covalently bound to the fluorescent protein, A.

The chemically reactive energy transfer compositions of the invention are useful for the preparation of any conjugated substance that possess a suitable functional group for covalent attachment of the fluorescent protein. Examples of particularly useful conjugates include, among others, conjugates of antigens, steroids, vitamins, drugs, haptens, metabolites, toxins, environmental pollutants, peptides, proteins, nucleic acids, nucleic acid polymers, carbohydrates, and non-biological polymers. Alternatively, these are conjugates of cells, cellular systems, cellular fragments, or subcellular particles. Examples include, among others, virus particles, bacterial particles, virus components, biological cells (such as animal cells, plant cells, bacteria, yeast, or protists), or cellular components. The reactive energy transfer compositions typically label reactive sites at the cell surface, in cell membranes, organelles, or cytoplasm. Preferably the conjugated substance is a peptide, protein, tyramine, polysaccharide, nucleic acid polymer, hapten, drug, hormone, ion

chelator, polymer, polymeric microparticle, biological cell or virus. In one embodiment, conjugates of biological polymers such as peptides, proteins, oligonucleotides, nucleic acid polymers are also labeled with a second fluorescent or non-fluorescent dye, including an additional dye of the present invention, to form an energy-transfer pair.

In one embodiment, the conjugated substance (S_c) is a polypeptide or protein. Preferred protein conjugates include enzymes, antibodies, lectins, glycoproteins, histones, albumins, lipoproteins, avidin, streptavidin, protein A, protein G, phycobiliproteins and other fluorescent proteins, hormones, toxins and growth factors. Typically, the conjugated polypeptide or protein is an antibody, an antibody fragment, an avidin, a streptavidin, a toxin, a lectin, a hormone, a cytokine, or a growth factor. Typically where the conjugated substance is a polypeptide, it is biologically active peptide, such as a neuropeptide or a toxin.

Where it is desired to label a nucleic acid polymer, typically a complex of the nucleic acid is formed with a fluorescent conjugate as described above. For example, a complex of a biotinylated nucleic acid with an APC-streptavidin. The nucleic acid polymer of the complex is single- or multi-stranded, natural or synthetic DNA or RNA, DNA or RNA oligonucleotides, or DNA/RNA hybrids, or incorporate an unusual linker such as morpholine derivatized phosphates, or peptide nucleic acids such as *N*-(2-aminoethyl)glycine units. When the nucleic acid is an oligonucleotide, it typically contains fewer than 50 nucleotides, more typically fewer than 25 nucleotides. Typically, the phycobiliprotein is attached via one or more purine or pyrimidine bases through an amide, ester, ether or thioether bond; or is attached to the phosphate or carbohydrate by a bond that is an ester, thioester, amide, ether or thioether. Alternatively, the conjugate of the invention is simultaneously labeled with a hapten such as biotin or digoxigenin, or to a protein such as an antibody. The nucleic acid polymer may be free in solution, in a cell, or partially immobilized such as in a gel, on a membrane, or on a gene chip. The nucleic acid may be a single molecule such as is detected using fluorescence correlation spectroscopy (FCS), or a collection of the same or different nucleic acid polymers.

In another embodiment, the conjugated substance (S_c) is a carbohydrate that is typically a monosaccharide or a polysaccharide, such as a dextran, FICOLL, heparin, glycogen, amylopectin, mannan, inulin, starch, agarose and cellulose. Alternatively, the carbohydrate is a polysaccharides that is a lipopolysaccharide.

Other conjugates of non-biological materials include dye-conjugates or dye-complexes of organic or inorganic polymers, polymeric films, polymeric wafers, polymeric membranes, polymeric particles, polymeric microparticles including magnetic and non-

magnetic microspheres. conducting and non-conducting metals and non-metals, and glass, silicon or plastic surfaces, particles and chips. Conjugates are typically prepared by chemical modification of a polymer that contains functional groups with suitable chemical reactivity, and complexes are typiclly formed by modification of the material with a hapten or biotin, and complexation with a dye-protein conjugate of the invention. In another embodiment, the conjugated substance is a glass or silica, which may be formed into an optical fiber or other structure.

The preparation of conjugates using reactive dyes is well documented, e.g. by MOLECULAR PROBES HANDBOOK, *supra*. Additionally, methods for preparation and use of fluorescent protein conjugates are well documented (for example U.S. Patent No. 5,055,556 to Stryer et al., *supra*; MOLECULAR PROBES HANDBOOK, *supra*, Chap. 6; U.S. Patent No. 4,876,190 to Recktenwald, *supra*; Haugland et al. "Coupling of Antibodies with Biotin" THE PROTEIN PROTOCOLS HANDBOOK, J.M. Walker, ed., Humana Press, (1996); Haugland "Coupling of Monoclonal Antibodies with Fluorophores" METHODS IN MOLECULAR BIOLOGY, VOL. 45: MONOCLONAL ANTIBODY PROTOCOLS, W.C. Davis, Ed. (1995)).

Labeled members of a specific binding pair are typically used as fluorescent probes for the complementary member of that specific binding pair, each specific binding pair member having an area on the surface or in a cavity that specifically binds to and is complementary with a particular spatial and polar organization of the other. Preferred specific binding pair members are proteins that bind non-covalently to low molecular weight ligands, such as biotin, drug-haptens and fluorescent dyes (such as an anti-fluorescein antibody). Representative specific binding pairs are shown in Table 3.

Table 3. Representative Specific Binding Pairs

antigen	antibody
biotin	avidin (or streptavidin or anti-biotin)
IgG*	protein A or protein G
drug	drug receptor
toxin	toxin receptor
carbohydrate	lectin or carbohydrate receptor
peptide	peptide receptor
protein	protein receptor
enzyme substrate	enzyme
DNA (RNA)	aDNA (aRNA)†
hormone	hormone receptor

* IgG is an immunoglobulin

† aDNA and aRNA are the antisense (complementary) strands used for hybridization

Applications and Methods of Use

The energy transfer compositions of the invention are useful as detection reagents and as fluorescent tracers in a wide variety of applications, most of which have been described for similar protein-based fluorescent dyes that do not have the useful spectral properties of the compositions of this invention. These useful spectral properties include a large effective Stokes shift when excited near the absorption maximum of the donor dye(s), combined with their high absorbance at this excitation wavelength. This large Stokes shift permits extremely sensitive detection with minimal autofluorescence or scattered light from either the excitation source or via Raman scattering. Furthermore these spectral properties facilitate multicolor applications such as in immunofluorescence (Examples 12 and 14), fluorescence *in situ* hybridization (FISH), staining of targets on surfaces, tracing, and related applications. This is particularly important in flow cytometry, where one frequently uses three or more dye conjugates to simultaneously stain and analyze biological cells (for example as in METHODS IN CELL BIOLOGY, Darzynkiewics et al., eds., Volume 41, Chapter 4 (1994)). Methods for qualitative and quantitative analysis of multicolor experiments are well documented in the art.

The care and handling of the instant energy transfer compositions are substantially the same as for well known phycobiliprotein labels (see for example, MOLECULAR PROBES HANDBOOK, *supra*, Section 6.4 and references therein).

The energy transfer compositions of the invention are generally utilized by combining a composition as described above with the sample of interest under conditions selected to yield a detectable optical response. The energy transfer composition typically forms a covalent or non-covalent association or complex with an element of the sample, or is

simply present within the bounds of the sample or portion of the sample. The sample is then illuminated at a wavelength selected to elicit the optical response. Typically, staining the sample is used to determine a specified characteristic of the sample by further comparing the optical response with a standard or expected response.

For biological applications, the compositions of the invention are typically used in an aqueous, mostly aqueous or aqueous-miscible solution prepared according to methods generally known in the art. The exact concentration of the composition is dependent upon the experimental conditions and the desired results, but typically ranges from about one nanomolar to one millimolar.

The composition is combined with the sample in any way that facilitates contact between the composition and the sample components of interest, including simple addition of the composition, or by treatments that permeabilize the plasma membrane (such as electroporation, osmotic shock treatments or high extracellular ATP), or by physical insertion (e.g. by pressure microinjection, scrape loading, patch clamp methods, or phagocytosis).

The sample is optionally washed after staining to remove residual, excess or unbound energy transfer compositions. The sample is optionally combined with one or more other solutions in the course of staining, including wash solutions, permeabilization and/or fixation solutions, and solutions containing additional detection reagents. An additional detection reagent typically produces a detectable response due to the presence of a specific cell component, intracellular substance, or cellular condition, according to methods generally known in the art. Where the additional detection reagent has, or yields a product with, spectral properties that differ from those of the subject dye compounds, multi-color applications are possible.

The compositions of the invention that are conjugates are used according to methods extensively known in the art; e.g. use of antibody conjugates in microscopy and immunofluorescent assays; and oligonucleotide conjugates for nucleic acid hybridization assays. For example, the fluorescent conjugates of the invention may be used as secondary detection reagents, for example by using goat-anti-mouse IgG to detect mouse antigens, while using streptavidin to detect biotinylated probes.

When used in combination with other dyes for multicolor applications (Examples 12 and 14) the other fluorescent labels are typically conjugates of other biopolymers such as of proteins or nucleic acid probes or they are lower molecular weight stains such as nucleic acid stains, organelle stains, products of fluorogenic enzyme substrates, probes for receptors, other tracers or other fluorescent labels.

At any time after or during staining, the sample is illuminated with a wavelength of light selected to give a detectable optical response, and observed with a means for detecting the optical response. Illumination sources include, but are not limited to, hand-held ultraviolet lamps, mercury arc lamps, xenon lamps, lasers and laser diodes. These illumination sources are optionally integrated into laser scanners, fluorescence microplate readers, standard or minifluorometers, or chromatographic detectors. Alternatively, the source of illumination is energy transfer from a chemiluminescent species.

A detectable optical response means a change in, or occurrence of, an optical signal that is detectable either by observation or instrumentally. Typically the detectable response is a change in fluorescence, such as a change in the intensity, excitation or emission wavelength distribution of fluorescence, fluorescence lifetime, fluorescence polarization, or a combination thereof. The degree and/or location of staining, compared with a standard or expected response, indicates whether and to what degree the sample possesses a given characteristic.

The optical response is optionally detected by visual inspection, or by use of any of the following devices: CCD cameras, video cameras, photographic film, laser-scanning devices, fluorometers, photodiodes, quantum counters, epifluorescence microscopes, scanning microscopes, flow cytometers, fluorescence microplate readers, or by means for amplifying the signal such as photomultiplier tubes. Where the sample is examined using a flow cytometer, examination of the sample optionally includes sorting components of the sample according to their fluorescence response.

This invention further comprises kits, wherein one or more kit components is a reagent of this invention. Additional kit components optionally comprise one or more of the following: additional detection reagents, buffers, fluorescence standards, and instructions for performing one or more assays using the kit. The fluorescence standards may comprise single fluorescent compounds, or preferably, fluorescent-labeled polymeric microspheres, more preferably microspheres labeled internally with fluorescent dyes.

The examples below are given so as to illustrate the practice of this invention. They are not intended to limit or define the entire scope of this invention.

EXAMPLES

Example 1. Conjugation of phycobiliproteins with synthetic dyes.

A solution of the desired fluorescent protein, such as a phycobiliprotein, is prepared at a concentration of 10 mg/mL in 0.1 M phosphate, 0.1 M NaCl, pH 7.5. The selected synthetic dye is dissolved in anhydrous DMF at a concentration of 10 mg/mL. The dye solution is then added to the fluorescent protein solution with stirring. The molar ratio of dye to protein in the reaction mixture is adjusted so as to yield a composition exhibiting relatively low residual fluorescence emission of the donor dye or donor dyes and relatively high fluorescence intensity of the fluorescent protein that is the FRET acceptor. The reaction mixture is incubated at room temperature for 1 hour, the reaction is stopped by addition of 1.5 M hydroxylamine, pH 8.0 in a volume corresponding to 1/10 of the volume of the reaction mixture. and the reaction mixture is incubated for an additional 30 minutes.

The reaction mixture is purified by size-exclusion chromatography on BioGel P-30 (BioRad). The degree of labeling (moles of dye per mole of protein) of the resulting compositions is measured using the extinction coefficient for the unconjugated donor dye and the protein (PRO) at their absorption maxima according to the following equation:

$$\text{Mol of dye/mol of PRO} = [(Abs_{dye} - CF)/E^{M}_{dye}] \times (E^{M}_{PRO}/Abs_{max} \text{ of PRO})$$

Where CF is the correction factor corresponding to the contribution by the unconjugated protein's absorption at the absorption maximum of the donor dye (s). This correction factor is determined as follows:

$$CF = (\text{Abs of PRO at } Abs_{dye}/ Abs_{max} \text{ of PRO})$$

The energy transfer compositions of Table 2 are prepared using this general synthetic method, by careful selection of appropriate synthetic dyes and fluorescent proteins. For instance when the synthetic dye is an ALEXA FLUOR 488 dye and the fluorescent protein is cross-linked allophycocyanin (XL-APC), the results in Figure 1 are obtained. Additional examples of the spectra of energy transfer compositions given for FITC–XL-APC in Figure 2, OREGON GREEN 488 dye–XL-APC in Figure 3, CY3 dye–XL-APC in Figure 4, OREGON GREEN 488 dye–R-PE in Figure 5, and ALEXA FLUOR 350 dye–R-PE in Figure 6.

Example 2. Conjugation of a transfer dye to a preformed donor dye-labeled phycobiliprotein.

A composition comprising ALEXA FLUOR 488 dye conjugated to XL-APC (Table 2) is dissolved in a pH 7.5 buffer (as in Example 1) and incubated with variable amounts of the succinimidyl ester of ALEXA FLUOR 568 dye. Following purification and characterization as described in Example 1, the resulting composition exhibits decreased residual fluorescence of the first donor dye (Figure 7). Alternatively the transfer dye(s) can be conjugated simultaneously with or preceding conjugation of the first donor dye.

Example 3. Preparation of conjugates of XL-APC with longer wavelength dyes, where the phycobiliprotein acts as donor.

A fluorescent protein that has been modified with a donor dye or dyes such as ALEXA FLUOR 488 dye can be further conjugated to a dye that has spectral overlap with the emission of the fluorescent protein but has overall a longer wavelength emission maximum. For instance, XL-APC that has been first modified by conjugation to ALEXA FLUOR 488 dye (Compound 4 of Table 1) can be further modified by CY7 dye (Compound 17 of Table 1) essentially as described in US Patent No. 5,268,486 to Waggoner et al. or with LaserPro 790 succinimidyl ester dye (Molecular Probes, Eugene OR), giving conjugates that can be excited at or near 488 nm with emission detected beyond ~700 nm.

Additionally, R-phycoerythrin that has been modified by CASCADE BLUE dye (Figure 8) can be further modified by conjugation to a TEXAS RED dye, e.g. such as described in US Patent 5,798,276 to Haugland et al. (1998) or with CY5 dye as described in US Patent No. 5,268,486 to give a conjugate that can be excited in the ultraviolet but that has principal fluorescence emission beyond ~600 nm (Figure 9).

Example 4. Conjugation of a low molecular weight ligand to a donor dye-fluorescent protein of this invention.

An ALEXA FLUOR 488 dye–XL-APC composition is biotinylated with biotin-X, succinimidyl ester (Molecular Probes) according to standard protocols ("Coupling of Antibodies with Biotin", Haugland et al., THE PROTEIN PROTOCOLS HANDBOOK, J.M. Walker ed., 1996, pg. 293). The conjugate is purified by size-exclusion column chromatography. The resulting complex can be combined with labeled or unlabeled avidins.

Example 5. Determination of FRET efficiency for the first donor dye.

The efficiency of energy transfer from the shortest wavelength emitting donor dye to the fluorescent acceptor protein is determined by comparing the fluorescence of the donor dye excited at or near its maximal absorption in the conjugate versus that of the same or a similar dye that is not conjugated to the protein. For instance, when ALEXA FLUOR 488 dye is conjugated to XL-APC to give an energy transfer composition (Table 1), the energy transfer efficiency for a particularly useful conjugate is typically above 95% (Figure 10). The conjugation of additional transfer dyes to the composition improves energy transfer efficiency to an even greater percentage.

Further confirmation that FRET is occurring, and therefore responsible for the improved long wavelength fluorescence of the conjugates is provided in Figure 11. The excitation spectra of the ALEXA FLUOR 488 dye–XL-APC composition features excitation bands for both the ALEXA FLUOR 488 fluorophore and the allophycocyanin, although the phycobiliprotein exhibits enhanced fluorescence emission.

Example 6. Emission efficiency of the acceptor protein.

The emission efficiency (quantum yield) of a fluorescent moiety is an indication of how much of the absorbed excitation energy is re-emitted as fluorescence. The emission efficiency of the fluorescent protein is not substantially altered by the conjugation of donor dyes. A comparison of the integrated areas of the fluorescence emission peaks of a solution of ALEXA FLUOR 488 dye–XL-APC and a solution of the free XL-APC, when excited at the maximum absorption wavelength of XL-APC, reveals that the energy transfer composition is nearly as fluorescent as the unconjugated protein (Figure 12). Both solutions are matched for absorption at the excitation wavelength. The chemical modification of the phycobiliprotein does not appreciably interfere with the protein's absorbance or fluorescence quantum yield.

Example 7. FRET efficiency as a function of degree of substitution.

FRET efficiency between the donor dye and the fluorescent protein may be measured by exciting the energy transfer composition at the optimal excitation wavelength of the donor dye and measuring the ratio of the long wavelength emission to that of the short wavelength emission. We have found that FRET efficiency varies as a function of the degree of substitution of the donor dye(s) (Figure 13). A high ratio is preferred so as to reduce spectral compensation in the conjugate's multicolor applications. Some results showing the ratio between the emission peak of the acceptor versus the residual emission peak of the donor, obtained by labeling B-PE, R-PE, APC and XL-APC with various non-

sulfonated and sulfonated dyes are reported in Table 2. From the results presented, it can be concluded that sulfonation of dyes yields donor dyes that are particularly advantageous for preparing the energy transfer compositions of the invention. Unexpectedly, such sulfonated donor dyes, besides typically having increased fluorescence emission relative to the non-sulfonated dyes, give more efficient FRET when conjugated to intrinsically fluorescent proteins such as phycobiliproteins

Example 8. FRET takes place efficiently without performing the conjugation of the dye with phycobiliproteins at high salt concentrations.

Conjugation of the sulfonated dye to the fluorescent protein need not be performed in high salt as specified in US Patent No. 5,272,257 to Gupta (1993). Conjugation of native allophycocyanin (APC) and crosslinked allophycocyanin (XL-APC) to the succinimidyl ester of carboxyfluorescein or to the succinimidyl ester of ALEXA FLUOR 488 dye is performed exactly as described in the Gupta patent in presence or absence of 10% sodium sulfate. The use of high salt (reportedly in order to expose more hydrophobic regions of the molecule) is not necessary, and may even be inhibitory, when conjugating the proteins to dyes. There is no improvement in FRET between carboxyfluorescein and either APC or XL-APC when the conjugation is performed in the presence of 10% sodium sulfate (Figure 14). However, at a high mole ratio of carboxyfluorescein to protein, native APC breaks down at a much lower degree of labeling than XL-APC, demonstrating the increased stability of the molecule when the subunits are chemically crosslinked (see Example 9).

Example 9. Conjugation of chemically cross-linked phycobiliproteins yields energy transfer compositions having advantageous spectral and chemical properties.

Energy transfer compositions comprising ALEXA FLUOR 488 dye and either native APC or cross-linked APC (XL-APC), respectively, are prepared using the procedure of Example 1, wherein the molar ratio of dye to protein is 30. A comparison of the absorption spectra of the two conjugates (Figure 15) shows that the composition containing native APC exhibits an altered absorption spectrum, having an absorption maximum at ~630 nm instead of ~650 nm. The composition containing XL-APC has maintained an absorption spectrum characteristic of the native protein, with an absorption maximum at ~650 nm. Furthermore, the relative fluorescence yield of the conjugate made from native APC is much lower than the relative yield of the conjugate prepared from XL-APC (Figure 15). Chemical crosslinking of the phycobiliprotein subunits permits the preparation of energy

transfer compositions that retain the integrity and high fluorescence intensity of native phycobiliprotein.

Example 10. Protein labeling using energy transfer compositions.

Fluorescent labeling of proteins that are specific binding members, such as an antibody, avidin, streptavidin, lectin, enzyme or the like, using the energy transfer compositions of the invention is accomplished by methods well known in the art. For instance, a labeled phycobiliprotein (as prepared in Examples 1, 2 or 3) may be further modified by conjugation of 1-2 moles of SPDP (3-(2-pyridyldithio)propionic acid succinimidyl ester), to give a pyridyldisulfide-modified protein. Following reduction of the disulfide, such as with dithiothreitol (DTT), the thiolated protein is readily conjugated to any thiol-reactive second protein, such as one that has been modified by SMCC (4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid succinimidyl ester). The labeled protein is then typically purified using size-exclusion chromatography. Covalent conjugates of molecules other than proteins, such as oligonucleotides, nucleic acids, microspheres, liposomes and so forth are readily prepared by similar methods, using the same or other crosslinking reagents known in the art.

Example 11. Using the energy transfer compositions to stain biological samples.

The use of specific binding pair members labeled with energy transfer compositions is typically analogous to well-known applications of the particular specific binding pair member used. The instant compositions are highly useful in applications such as staining cells, blots, targets immobilized on DNA chips and the like.

Permeabilized and fixed bovine pulmonary artery endothelial cells (BPAEC), grown on coverslips, are reacted with 1 μg/mL mouse monoclonal anti-α tubulin. The cells are washed and treated with 10 μg/mL of biotinylated goat anti-mouse IgG and with streptavidin-modified ALEXA FLUOR 488 dye–XL-APC at 5 μg/mL. Tubulin filaments are stained well and can be excited with the fluorescein excitation filter at 490 +/-10 nm, and visualized with the longer wavelength emission filter of 660 +/-25 nm, which is appropriate for APC conjugates. The energy transfer composition can also be excited at about 620-650 nm. Negligible fluorescence from the donor dye is observed with excitation at ~490 nm.

Example 12. Multicolor flow cytometry with a single excitation wavelength.

Some of the energy transfer compositions of the invention and their conjugates are particularly useful for multiparametric flow cytometric applications, especially for those that use excitation by a single argon-ion laser at 488 nm or 514 nm.

To 1 million Jurkat cells is added 10 μL of T11-FITC (Cytostat Coulter # 6603863; 10 μL/test), 5 μL of T4-RPE (Coulter #6602864; 5 μL/test) and 10 μL of biotinylated anti-CD3 (Immunotech Coulter #1301). After 30 minutes on ice, the cells are washed twice using the wash buffer then streptavidin-modified ALEXA FLUOR 488 dye–XL-APC (1 μg/mL) is added. The cells are incubated for an additional 30 minutes on ice and analyzed by flow cytometry using appropriate single-color controls and negative controls. Analysis of dot plots clearly demonstrates the utility of streptavidin-modified ALEXA FLUOR 488 dye–XL-APC in performing three color analysis using a single laser for excitation. The fluorescent signal from a dot stained with the energy transfer composition is approximately 42 times brighter than a similar dot stained with streptavidin-modified XL-APC, when both spots are excited at 450 nm.

Example 13. Comparison of cell staining by streptavidin-modified ALEXA FLUOR 488 dye–XL-APC versus streptavidin-RED 670.

Two million Jurkat cells are stained with 20 μL of biotinylated anti-CD 4 Biotin (BioSource International #AHS0419; 10 μL/test). Following a 30-minute incubation on ice, the cells are washed twice with wash buffer and re-suspended in 200 μL of the same. To 100 μL of the cell suspension is added either 16 μL streptavidin-RED 670 (Gibco #19543-024; 0.25 mg/mL) or 3.6 μL of streptavidin-modified ALEXA FLUOR 488 dye–XL-APC (1.05 μg/mL). After incubation on ice for 30 minutes the cells are washed twice and analyzed by flow cytometry. The cells stained with streptavidin-modified ALEXA FLUOR 488 dye–XL-APC exhibit fluorescent staining that is approximately 120% brighter than those stained with streptavidin-RED 670 (Figure 16).

Example 14. Staining cells in combination with a nucleic acid stain.

One million Jurkat cells are incubated on ice for 30 minutes with 5 μL of T4-RD1, an R-PE labeled probe for the cell surface receptor CD4 (Coulter #6602864; 5 μL/test), and 10 μL of biotinylated anti-CD3 (0.2 mg/mL). After washing twice with wash buffer, streptavidin-modified ALEXA FLUOR 488 dye–XL-APC is added and the cells are incubated for an additional 30 minutes on ice. Following two washes, 0.2 μM SYTOX GREEN nucleic acid stain (Molecular Probes, Inc., Eugene OR) is added in order to stain cells having compromised membranes. The cells are then analyzed using flow cytometry

using appropriate single-color controls and negative controls. The dot plots shown in Figure 17 demonstrate the utility of the energy transfer compositions of the invention for performing three-color analyses involving a nucleic acid stain and two surface markers.

It is to be understood that, while the foregoing invention has been described in detail by way of illustration and example, numerous modifications, substitutions, and alterations are possible without departing from the spirit and scope of the invention as described in the following claims.

What is claimed is:

1. An energy transfer composition comprising

an intrinsically fluorescent protein, A, having a molecular weight less than 500,000;

wherein A has been stabilized by cross-linking; has a fluorescence emission maximum ($\lambda_{Em\text{-}max}$) greater than 450 nm; and an absorption maximum ($\lambda_{Ab\text{-}max}$); and

from 1 to 30 molecules of a fluorescent moiety, D, each covalently bound to A;

wherein D has a molecular weight less than 2,000; and a fluorescence emission maximum ($\lambda_{Em\text{-}max}$) at a shorter wavelength than the fluorescence emission maximum ($\lambda_{Em\text{-}max}$) of A.

2. An energy transfer composition comprising

an intrinsically fluorescent protein, A, having a molecular weight less than 500,000;

wherein A has a fluorescence emission maximum ($\lambda_{Em\text{-}max}$) greater than 450 nm; and an absorption maximum ($\lambda_{Ab\text{-}max}$); and

from 1 to 30 molecules of a fluorescent moiety, D, each covalently bound to A;

wherein D is substituted one or more times by $-SO_3X$, where X is H or a counterion; D has a molecular weight less than 2,000; and a fluorescence emission maximum ($\lambda_{Em\text{-}max}$) at a shorter wavelength than the fluorescence emission maximum ($\lambda_{Em\text{-}max}$) of A.

3. A composition, as claimed in Claim 1 or 2, wherein A is a phycobiliprotein.

4. A composition, as claimed in Claim 1 or 2, wherein A is a phycoerythrin, a phycocyanin or an allophycocyanin.

5. A composition, as claimed in Claim 1 or 2, wherein A is an allophycocyanin.

6. A composition, as claimed in Claim 1 or 2, wherein the $\lambda_{Em\text{-}max}$ of A is greater than 560 nm.

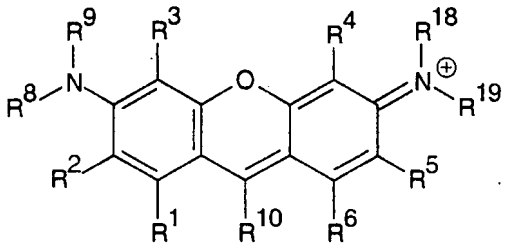
7. A composition, as claimed in Claim 1 or 2, wherein the $\lambda_{Em\text{-}max}$ of D is at least 25 nm less than the $\lambda_{Ab\text{-}max}$ of A.

8. A composition, as claimed in Claim 1 or 2, wherein the $\lambda_{Ab\text{-}max}$ of D lies between 480 nm and 520 nm.

9. A composition, as claimed in Claim 1 or 2, wherein D is a naphthalene, an anthracene, a pyrene, a stilbene, a coumarin, a carbostyryl, a pyridine, a quinoline, an acridine, a fluorescein, a rhodol, a rhodamine, a cyanine, a polyazaindacene, an oxazine, or a styryl dye.

10. A composition, as claimed in Claim 1 or 2, wherein D is a fluorescein, a rhodol, a rhodamine, a cyanine, or an oxazine.

11. A composition, as claimed in Claim 1 or 2, wherein each D has the formula



wherein

R^2, R^3, R^4 and R^5 are independently H, F, Cl, Br, I, CN; or C_1-C_{18} alkyl, or C_1-C_{18} alkoxy, where each alkyl or alkoxy is optionally further substituted by F, Cl, Br, I, a carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C_1-C_6 alcohol; or $-SO_3X$ where X is H or a counterion; or form fused rings as below;

R^1 and R^6 are H; or R^1 taken in combination with R^2, or R^5 taken in combination with R^6, or both, form a fused aromatic six membered ring that is optionally substituted one or more

times by $-SO_3X$;

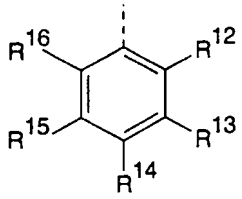
R^8 and R^9 are independently H, C_1-C_6 alkyl, C_1-C_6 carboxyalkyl, C_1-C_6 sulfoalkyl, a salt of C_1-C_6 carboxyalkyl, or a salt of C_1-C_6 sulfoalkyl, wherein the alkyl portions are optionally substituted by amino, hydroxy, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C_1-C_6 alkyl; or R^8 in combination with R^9 forms a saturated 5- or 6-membered heterocycle that is a piperidine, a morpholine, a pyrrolidine or a piperazine, each of which is optionally substituted by methyl, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C_1-C_6 alkyl;

or R^8 in combination with R^2, or R^9 in combination with R^3, or both, form a 5- or 6-membered ring that is saturated or unsaturated, and is optionally substituted by one or more C_1-C_6 alkyls or $-CH_2SO_3X$ moieties;

where R^{18} and R^{19} are independently H, C_1-C_6 alkyl, C_1-C_6 carboxyalkyl, C_1-C_6 sulfoalkyl, a salt of C_1-C_6 carboxyalkyl, or a salt of C_1-C_6 sulfoalkyl, wherein the alkyl portions are optionally substituted by amino, hydroxy, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C_1-C_6 alkyl; or R^{18} in combination with R^{19} forms a saturated 5- or 6-membered heterocycle that is a piperidine, a morpholine, a pyrrolidine or a piperazine, each of which is optionally substituted by methyl, carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C_1-C_6 alkyl;

or R^{18} in combination with R^4, or R^{19} in combination with R^5, or both, form a 5- or 6-membered ring that is saturated or unsaturated, and is optionally substituted by one or more C_1-C_6 alkyls or $-CH_2SO_3X$ moieties;

R^{10} is H, F, CN, a carboxylic acid, a salt of carboxylic acid, or a carboxylic acid ester of a C_1-C_6 alcohol; or R^{10} is a saturated or unsaturated C_1-C_{18} alkyl that is optionally substituted one or more times by F, Cl, Br, carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of a C_1-C_6 alcohol, $-SO_3X$, amino, alkylamino, or dialkylamino, the alkyl groups of which have 1-6 carbons; or R^{10} has the formula

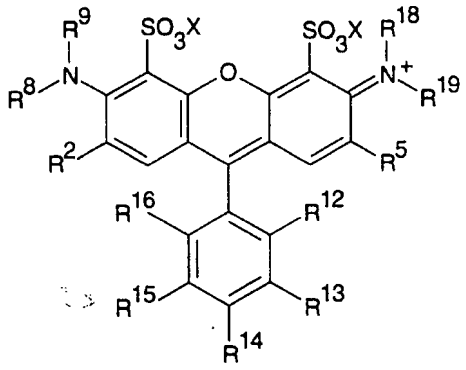


where R^{12}, R^{13}, R^{14}, R^{15} and R^{16} are independently H, F, Cl, Br, I, $-SO_3X$, a carboxylic acid, a salt of carboxylic acid, CN, hydroxy, amino, hydrazino; or C_1-C_{18} alkyl, C_1-C_{18} alkoxy, C_1-C_{18} alkylthio, C_1-C_{18} alkanoylamino, C_1-C_{18} alkylaminocarbonyl, C_2-C_{36} dialkylaminocarbonyl, C_1-C_{18} alkyloxycarbonyl, or C_6-C_{18} arylcarboxamido, the alkyl or aryl portions of which are optionally substituted one or more times by F, Cl, Br, I, hydroxy, carboxylic acid, a salt of carboxylic acid, a carboxylic acid ester of a C_1-C_6 alcohol, $-SO_3X$, amino, alkylamino, dialkylamino or alkoxy, the alkyl portions of each having 1-6 carbons; or one pair of adjacent substituents R^{13} and R^{14}, R^{14} and R^{15} or R^{15} and R^{16}, when taken in combination, form a fused 6-membered aromatic ring that is optionally further substituted by carboxylic acid, or a salt of carboxylic acid; and

provided that at least one of R^2, R^3, R^4, and R^5 is $-SO_3X$; or R^8 in combination with R^2, or R^9 in combination with R^3, or R^{18} in combination with R^4, or R^{19} in combination with R^5, form a 5- or 6-membered ring that is saturated or unsaturated, and is substituted by at least one $-CH_2SO_3X$ moiety;

provided D is attached to A via a covalent linkage L at one of R^2, R^3, R^4, R^5, R^8, R^9, R^{10}, R^{12}, R^{13}, R^{14}, R^{15}, or R^{16}.

12. A composition, as claimed in Claim 1 or 2, wherein each D has the formula



wherein

R^2 and R^5 are independently H, F, Cl, Br, I, C_1-C_{18} alkyl, or C_1-C_{18} alkoxy;

R^8, R^9, R^{18}, and R^{19} are independently H, C_1-C_6 alkyl, C_1-C_6 carboxyalkyl, a salt of C_1-C_6 carboxyalkyl, C_1-C_6 sulfoalkyl, or a salt of C_1-C_6 sulfoalkyl;

R^{12} is a carboxylic acid or a salt of carboxylic acid;

R^{13} and R^{16} are independently H, Cl, or F;

one of R^{14} and R^{15} is -L-A, and the other of R^{14} and R^{15} is H, Cl, or F;

provided that each D is substituted at one of R^{14} and R^{15} by -L-A;

where L is a covalent linkage having 1-20 nonhydrogen atoms selected from the group consisting of C, N, O, P, and S; and is composed of any combination of ether, thioether, amine, ester, carboxamide, sulfonamide, hydrazide bonds and aromatic or heteroaromatic bonds; and

A is an allophycocyanin.

13. A composition, as claimed in Claim 1 or 2, further comprising 1 to 10 additional fluorescent moieties that are chemically distinct from D, wherein each additional fluorescent moiety is present as 1 to 30 individual fluorophores that are covalently bound to

A; and for at least one additional fluorescent moiety, both the $\lambda_{Ab\text{-}max}$ and the $\lambda_{Em\text{-}max}$ are greater than the $\lambda_{Em\text{-}max}$ of A and less than the $\lambda_{Ab\text{-}max}$ of D.

14. A composition, as claimed in Claim 12, wherein each additional fluorescent moiety is a fluorescein, a rhodol, a rhodamine, a cyanine, a polyazaindacene, or an oxazine dye.

15. A composition, as claimed in Claim 14, wherein at least one additional fluorescent moiety is substituted one or more times by $-SO_3X$, where X is H or a counterion.

16. A composition, as claimed in Claim 1 or 2, further comprising a conjugated substance, S_c, that is covalently bound to A.

17. A composition, as claimed in Claim 16, wherein S_c is present as a plurality of substantially identical molecules, each covalently bound to A.

18. A composition, as claimed in Claim 16, wherein S_c is a peptide, a protein, an oligonucleotide, a nucleic acid, a polysaccharide, a biotin, a liposome, a polymeric film, or a polymeric microsphere.

19. A composition, as claimed in Claim 16, wherein S_c is an antibody, an avidin, a protein A, a protein G, an enzyme, a lectin, a cytokine, a hormone, or an additional intrinsically fluorescent protein having a $\lambda_{Em\text{-}max}$ greater than 450 nm.

20. A composition, as claimed in Claim 16, wherein S_c is a hapten, a natural or synthetic drug, or a solid or semi-solid matrix.

21. A composition, as claimed in Claim 1 or 2, further comprising 1-5 chemically reactive functional groups, R_x, covalently bound to A via a covalent linkage L, wherein each R_x has the same chemical structure.

22. A composition, as claimed in Claim 21, wherein each R_x is an acrylamide, an activated ester of a carboxylic acid, hydroxy, an aldehyde, an alkyl halide, a sulfonate ester, an amine, an anhydride, an aniline, an aryl halide, an azide, an aziridine, a boronate, a carboxylic acid, an epoxide, a glycol, a haloacetamide, a halotriazine, a hydrazine, a hydroxylamine, an isothiocyanate, a ketone, a maleimide, a thiol, or a disulfide group.

23. A method of staining a sample, comprising adding to said sample an energy transfer composition of any of claims 1-22 in a concentration sufficient to yield a detectable optical response under the desired conditions.

24. A method, as claimed in Claim 23, further comprising the steps of illuminating said sample at a wavelength selected to elicit said optical response; and detecting said optical response.

25. A method, as claimed in Claim 24, wherein said optical response is detected visually, photographically, or by using a fluorometer, a microscope, or a flow cytometer.

26. A method, as claimed in Claim 23, further comprising adding 1-3 additional detection reagents, each having an optical response that is detectably distinct from that of the energy transfer composition, and from each other.

27. A method, as claimed in Claim 23, wherein said energy transfer composition further comprises a conjugated substance, S_c, that is covalently bound to A, wherein S_c is a specific binding pair member; further comprising the step of correlating the location of said optical response with the presence of a complementary specific binding pair member.

28. A method, as claimed in Claim 23, wherein said sample is immobilized on a solid or semi-solid matrix.

29. A method, as claimed in Claim 28, wherein said matrix is a nitrocellulose membrane, a glass, or a silicon surface.

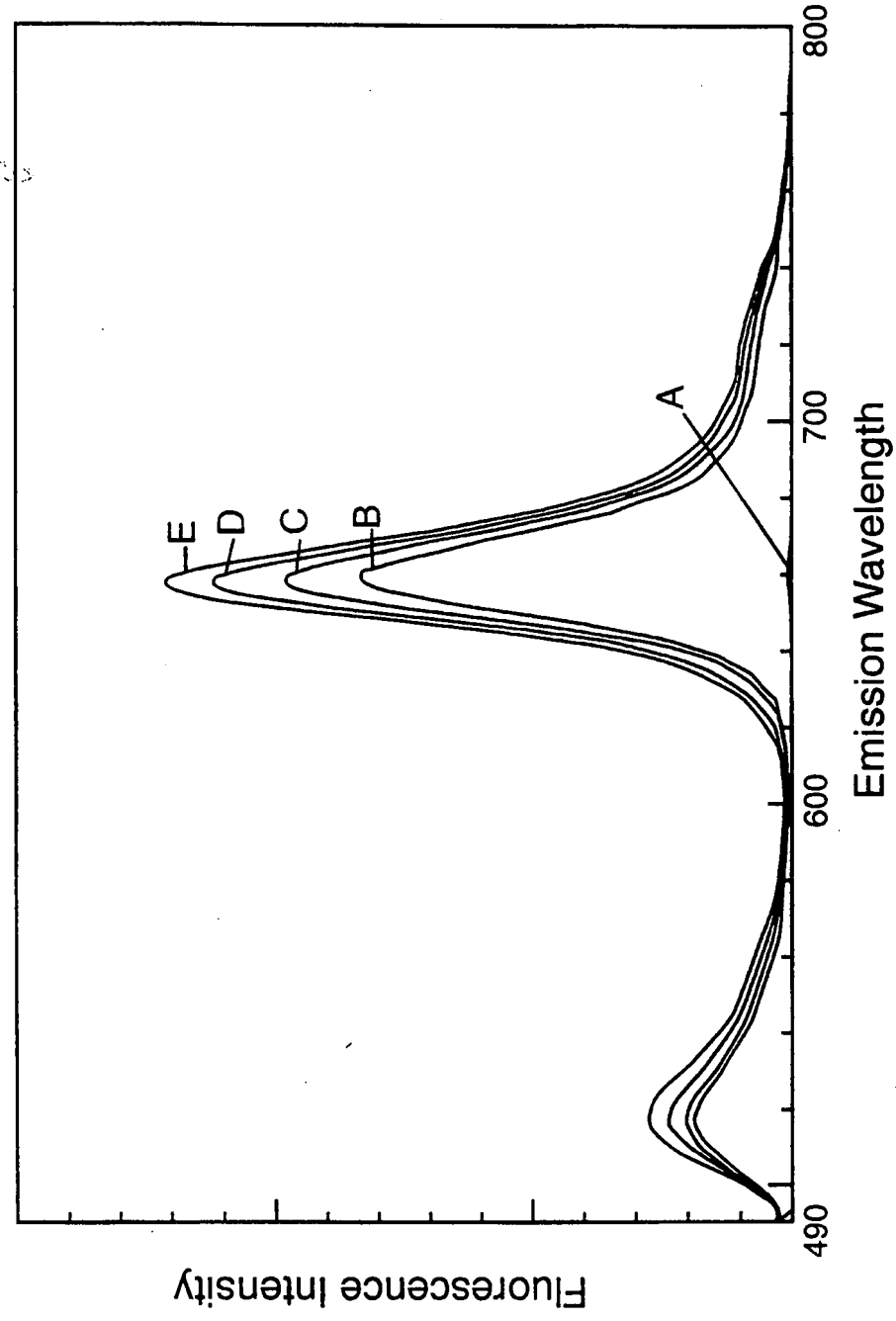
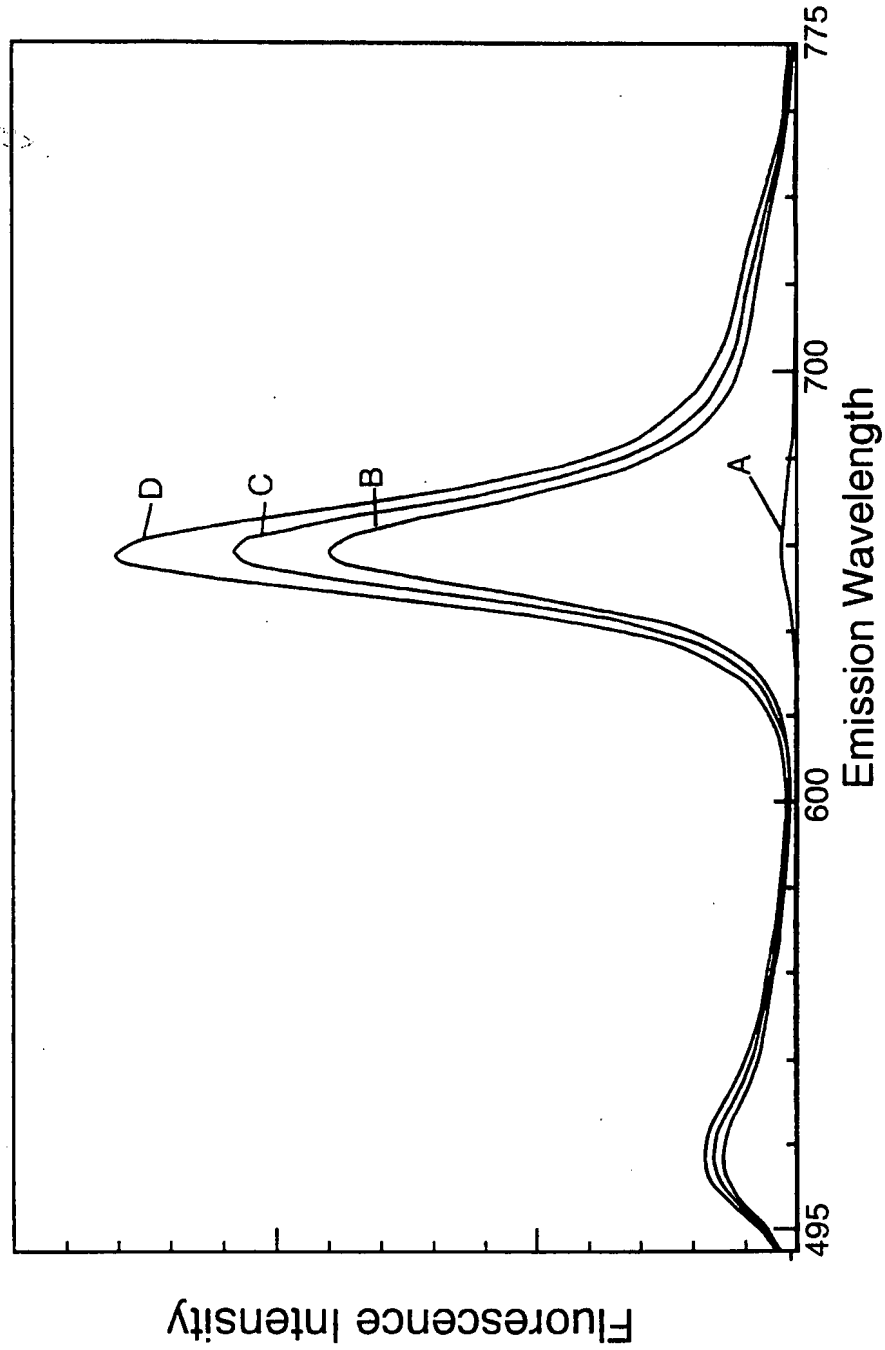


Figure 1



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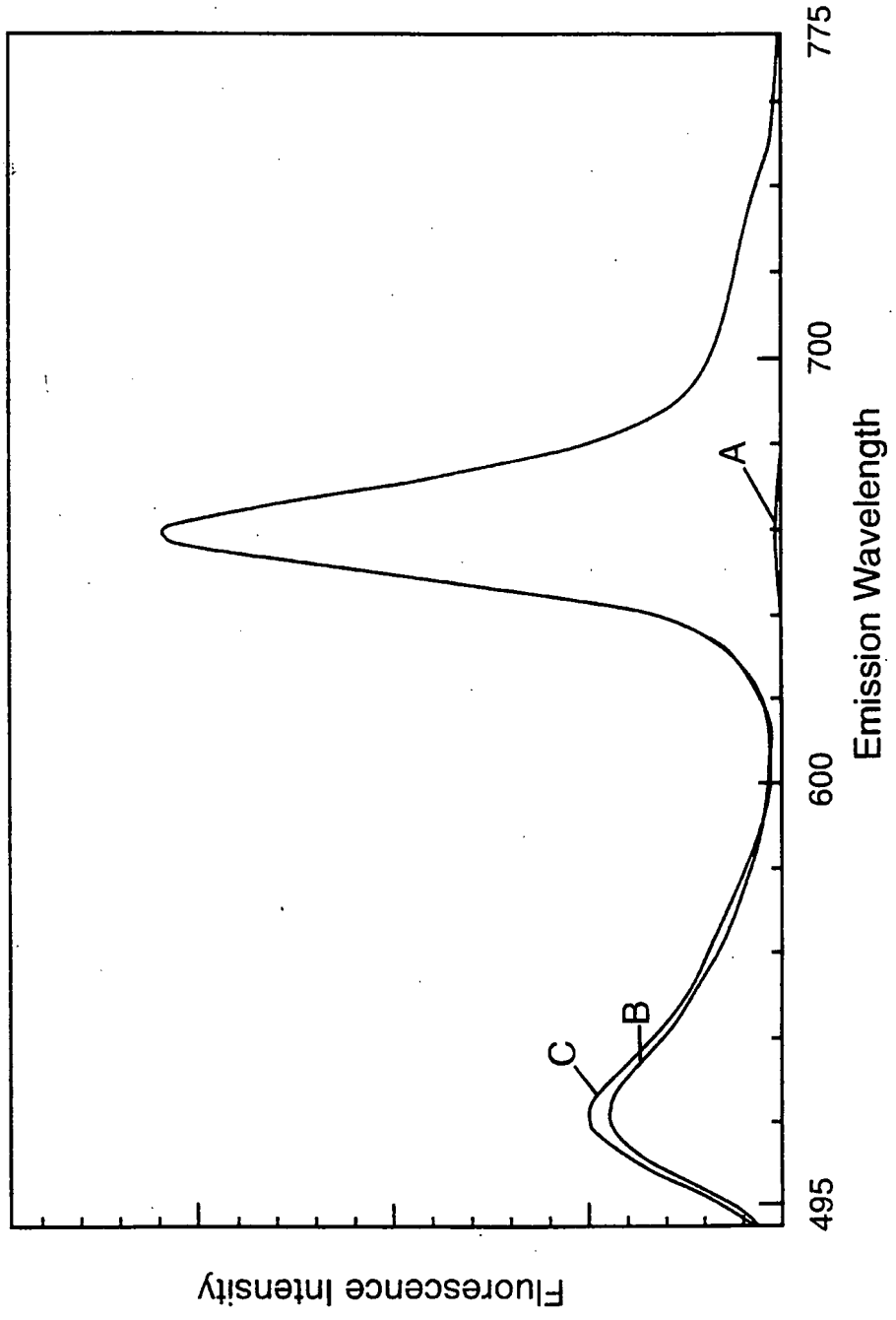

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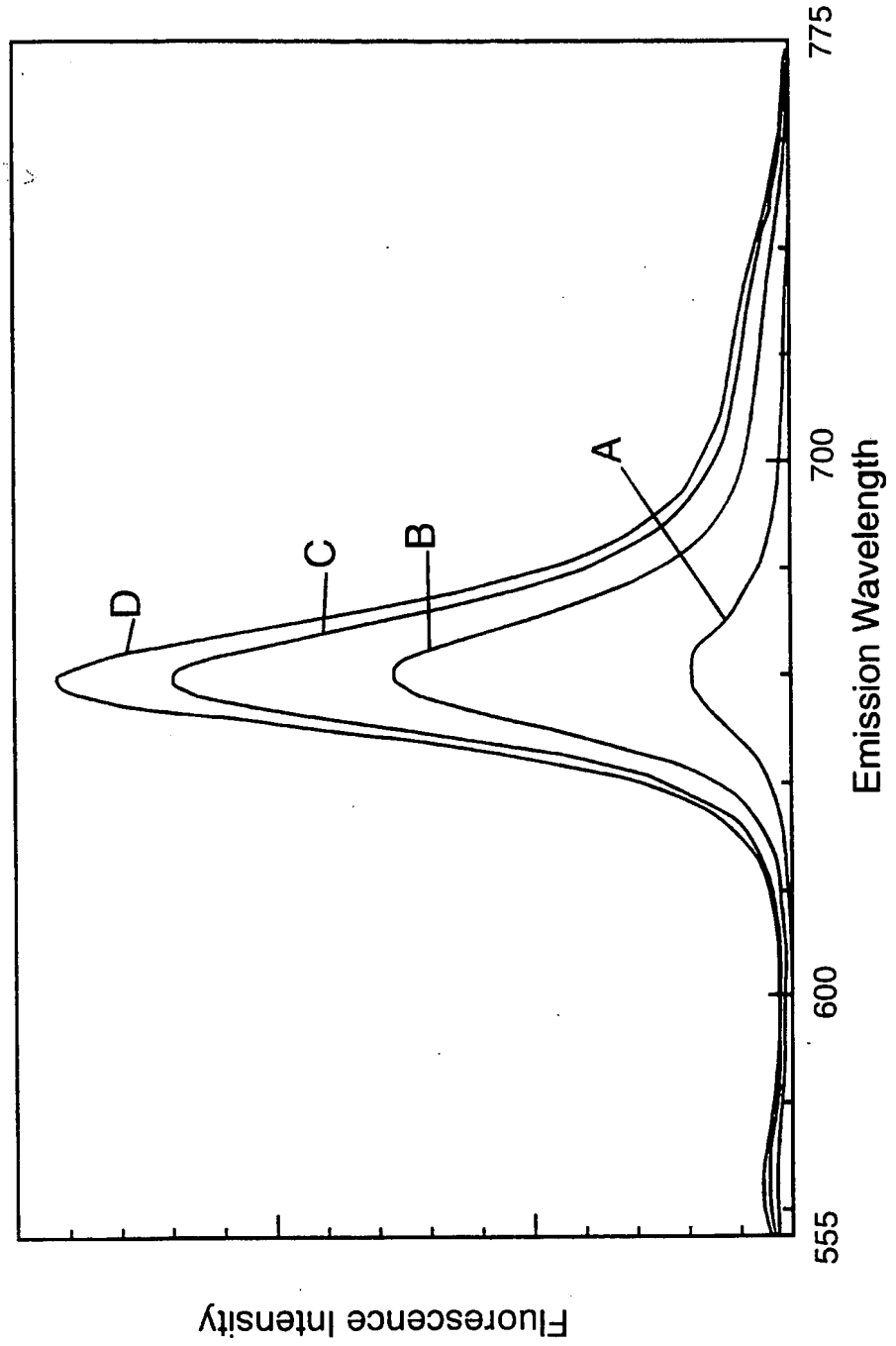

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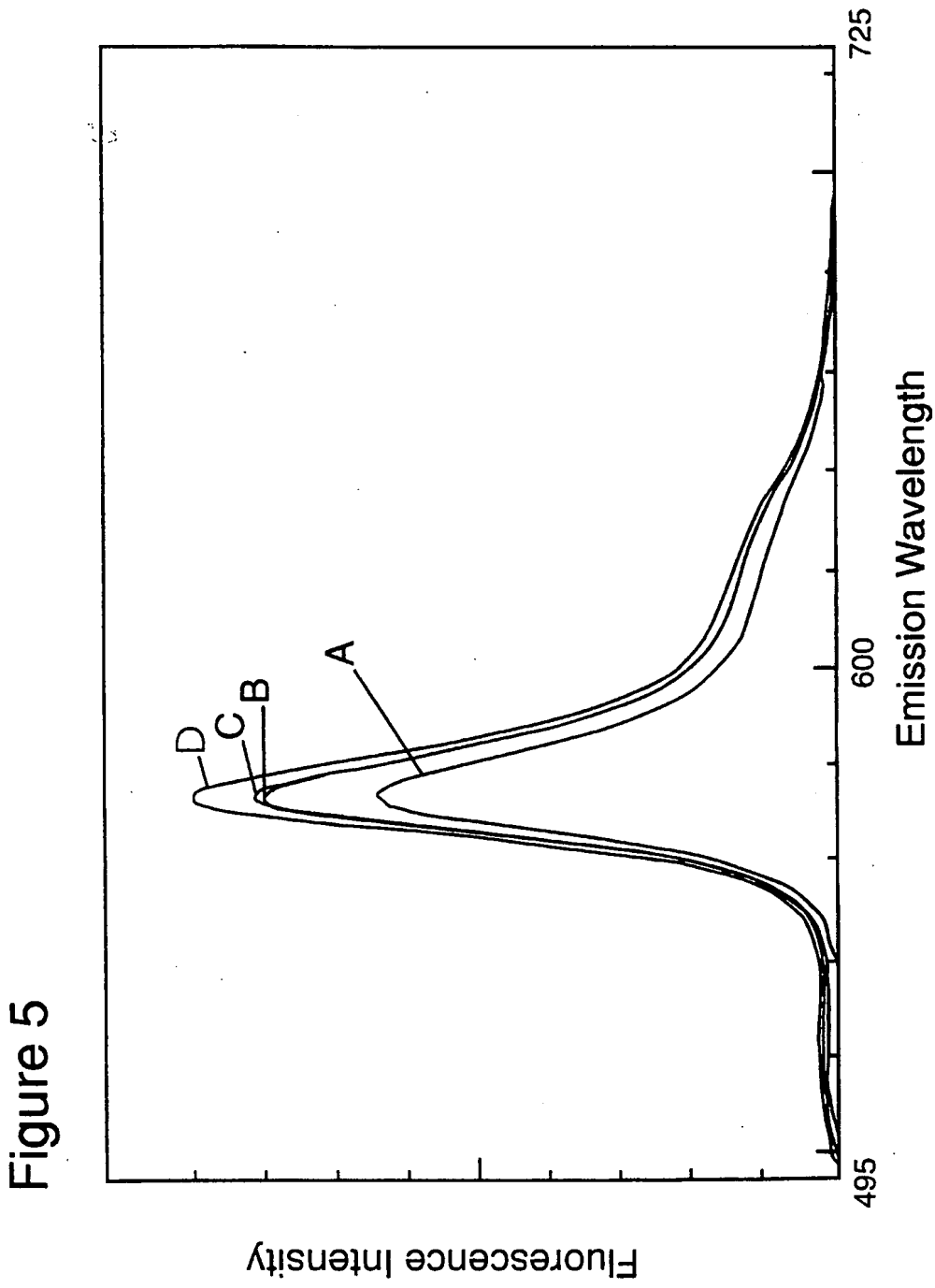

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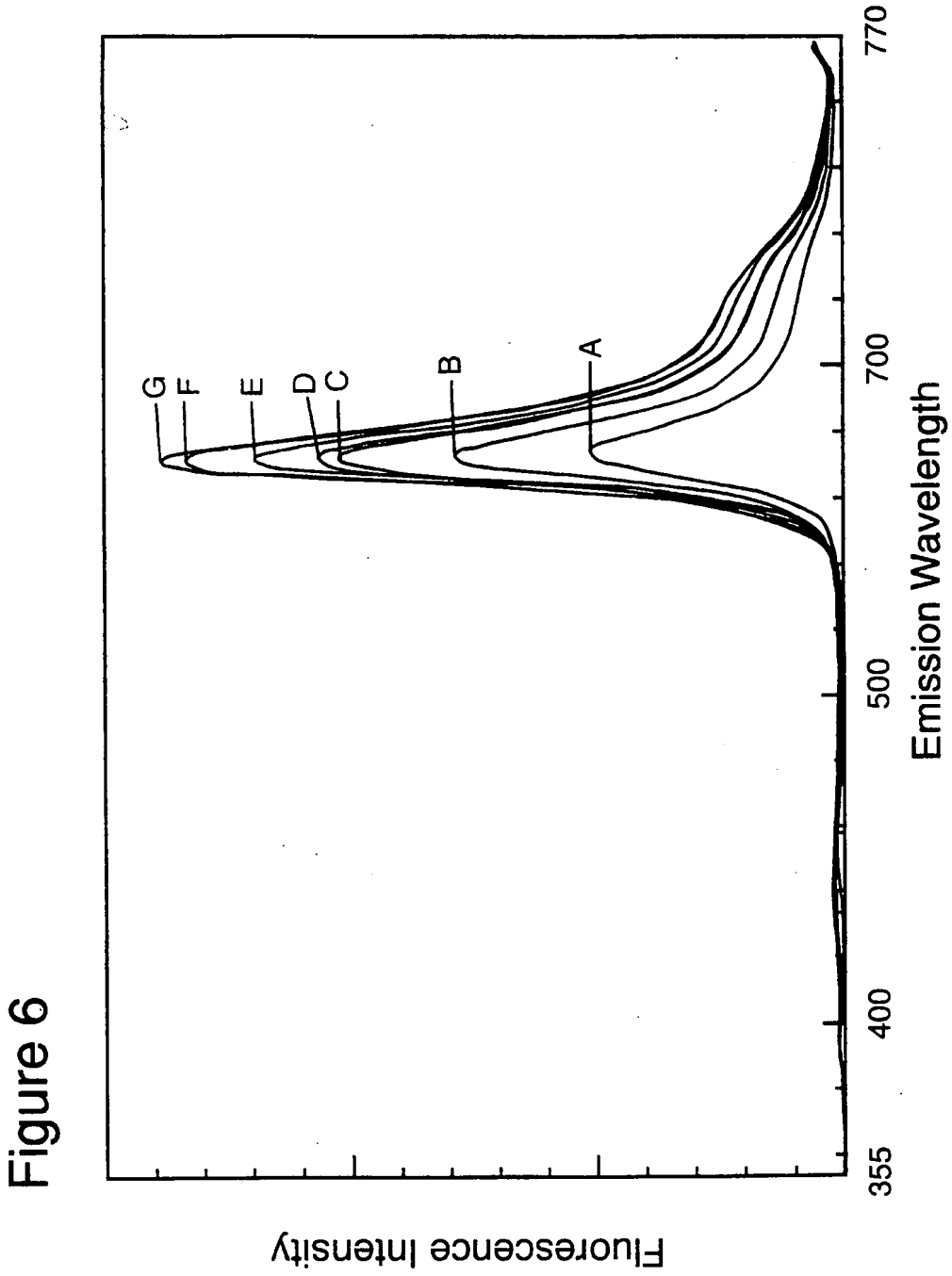

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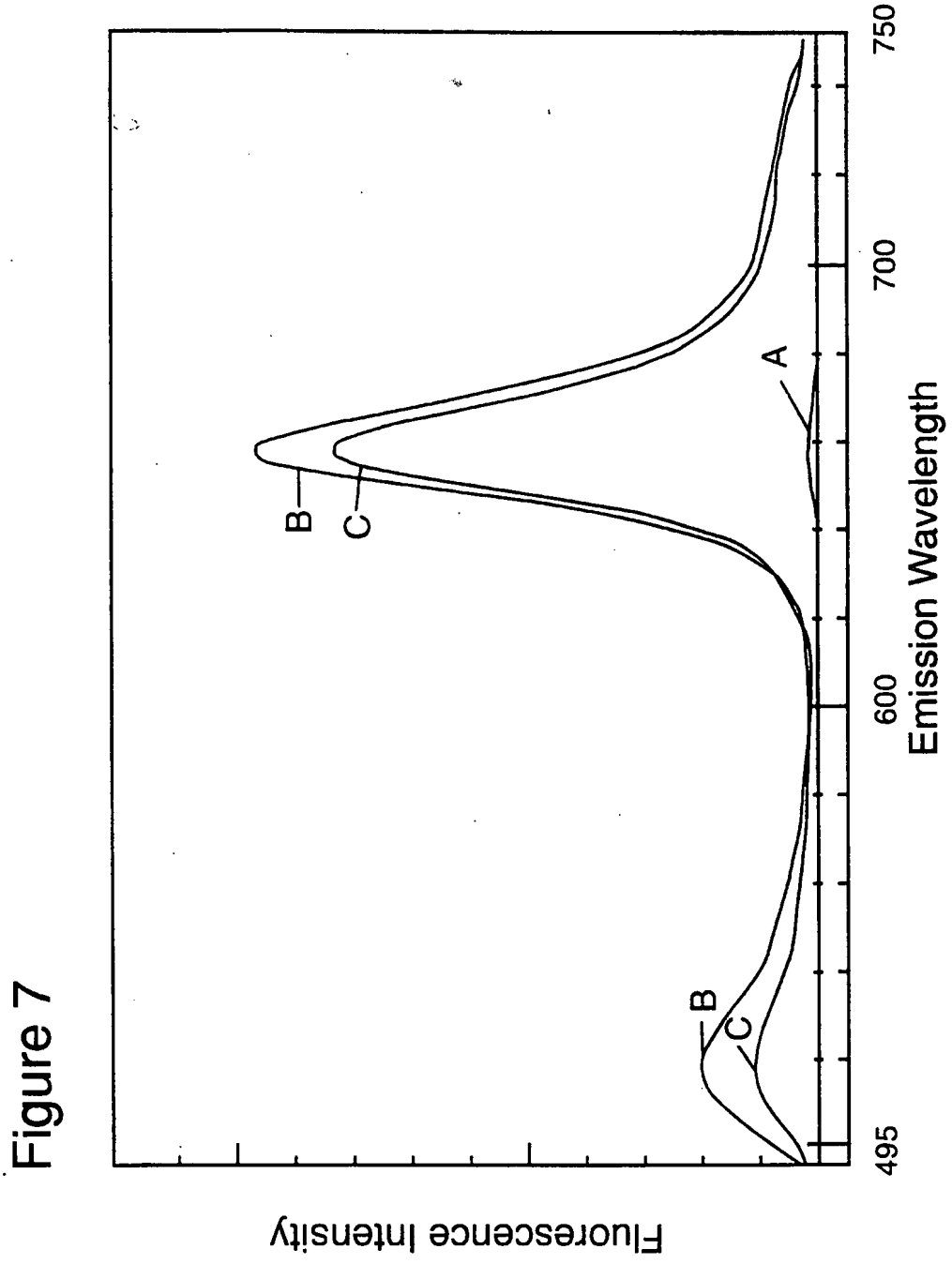

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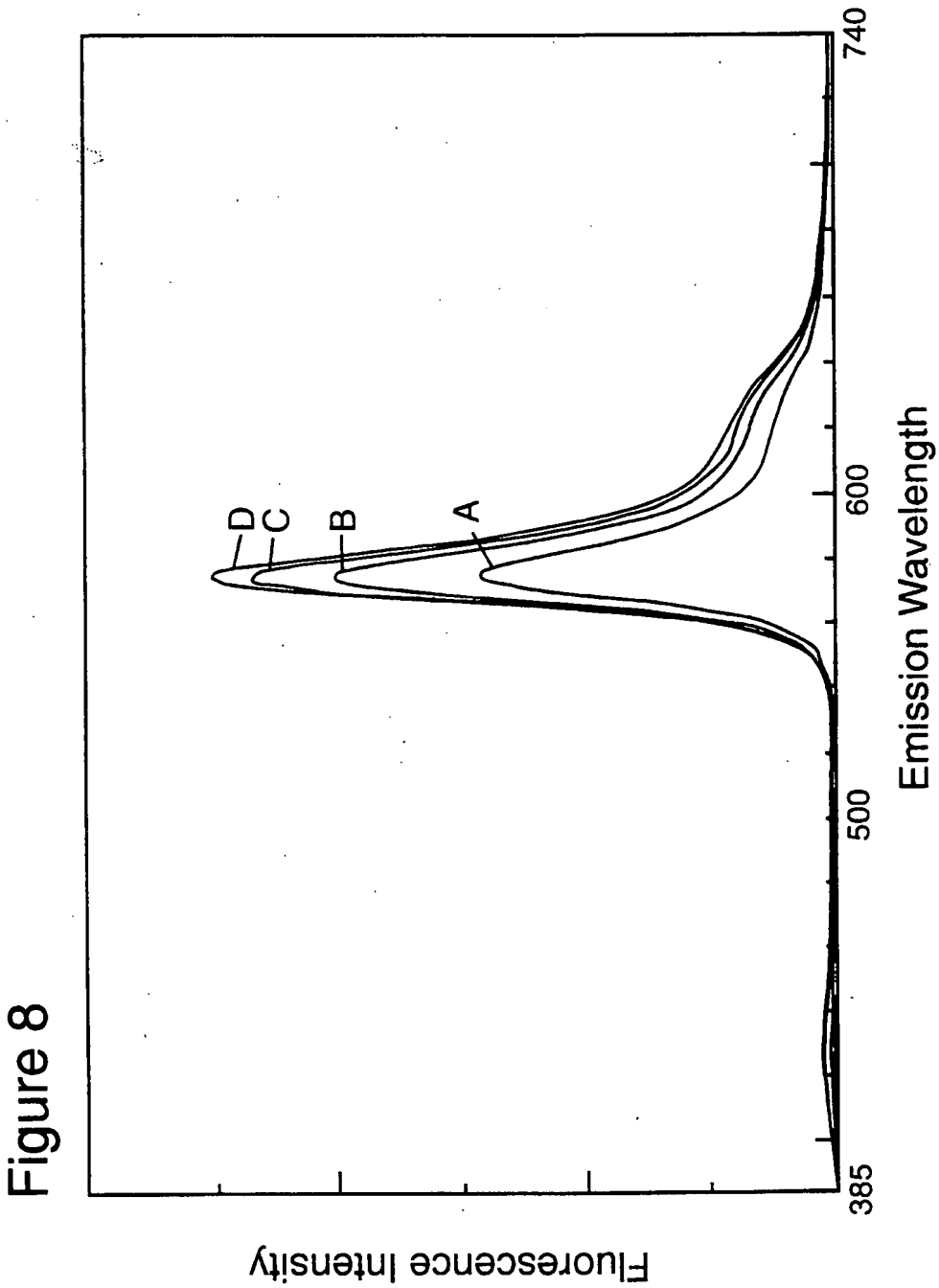

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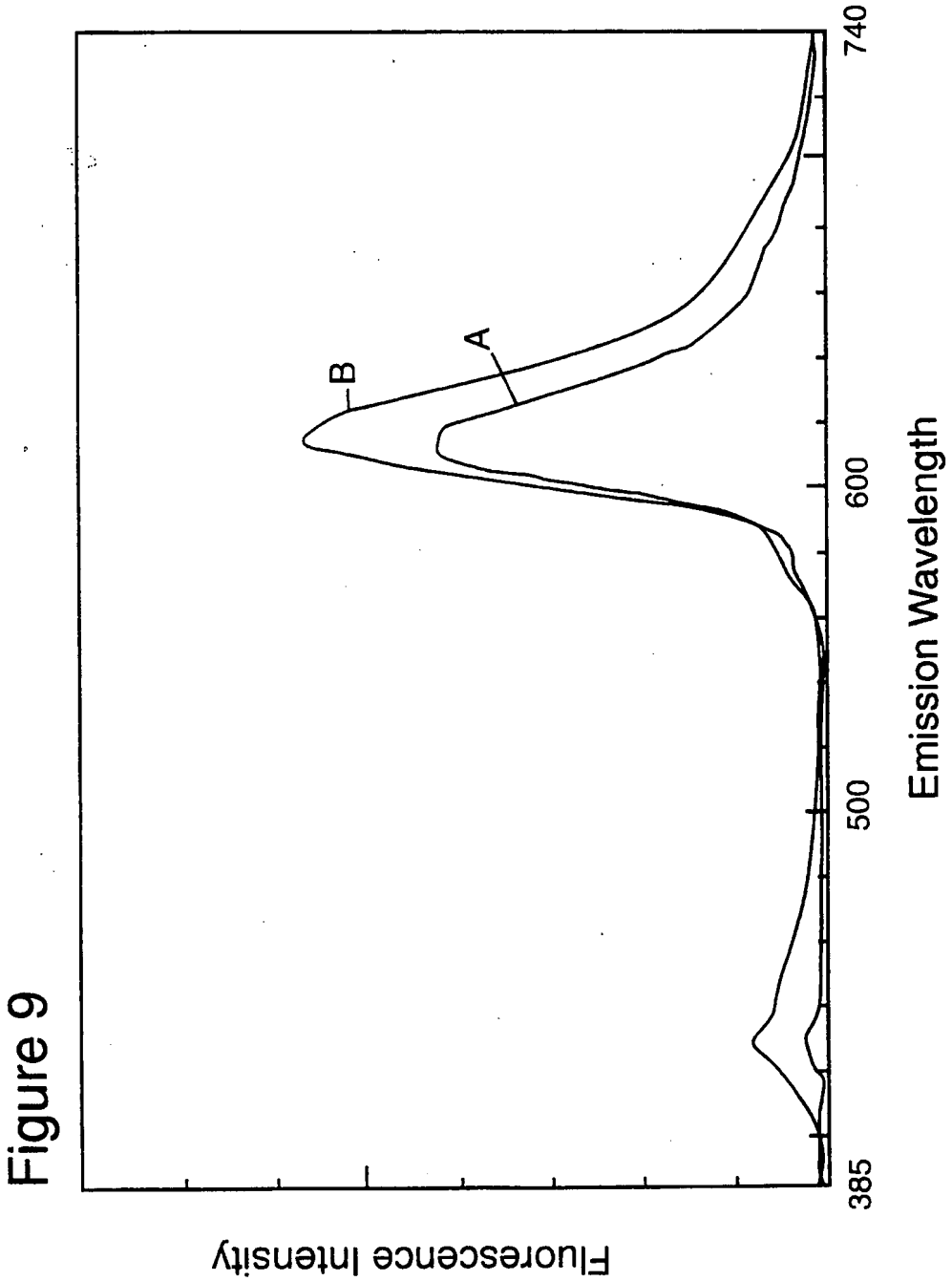

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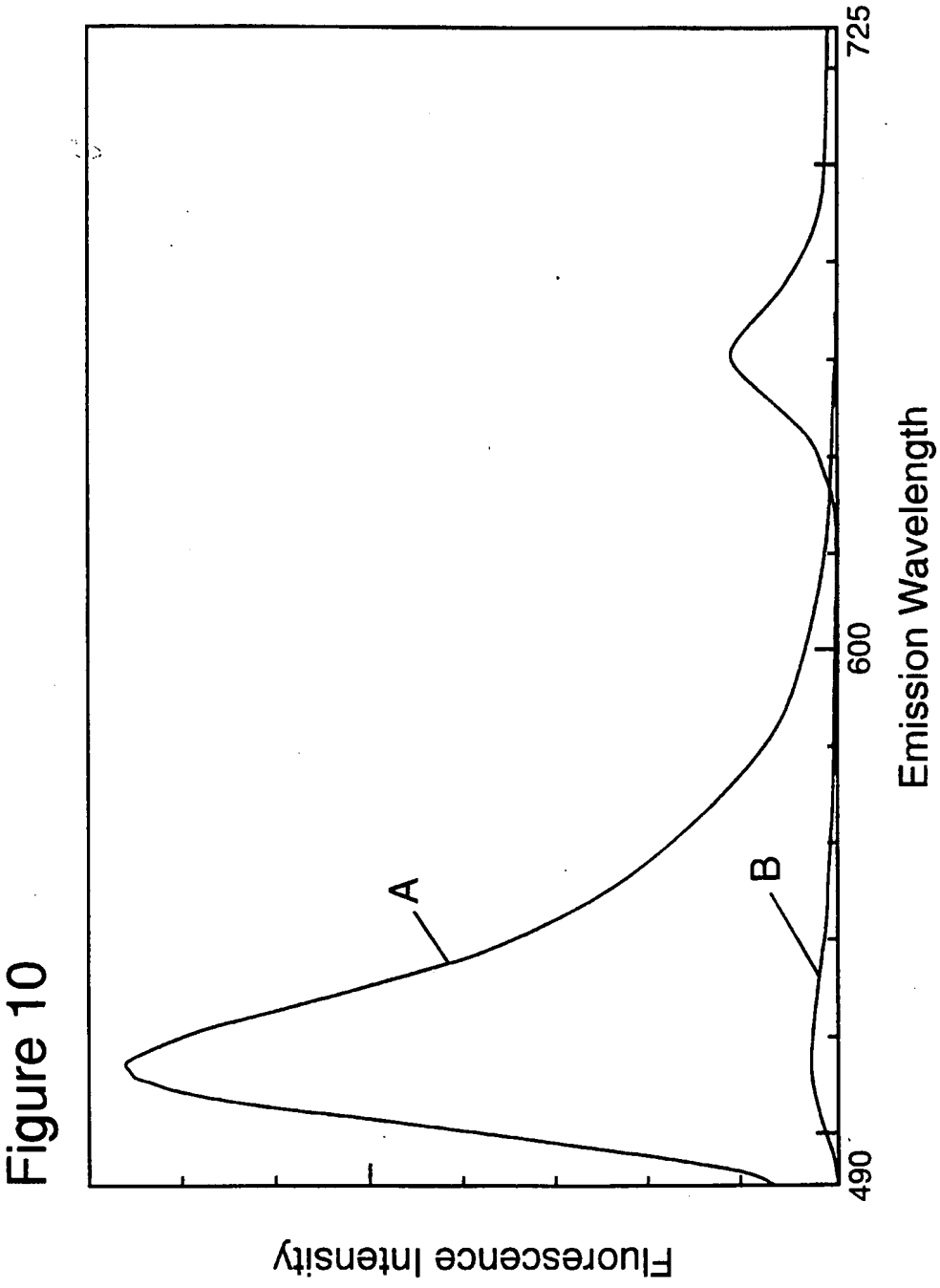

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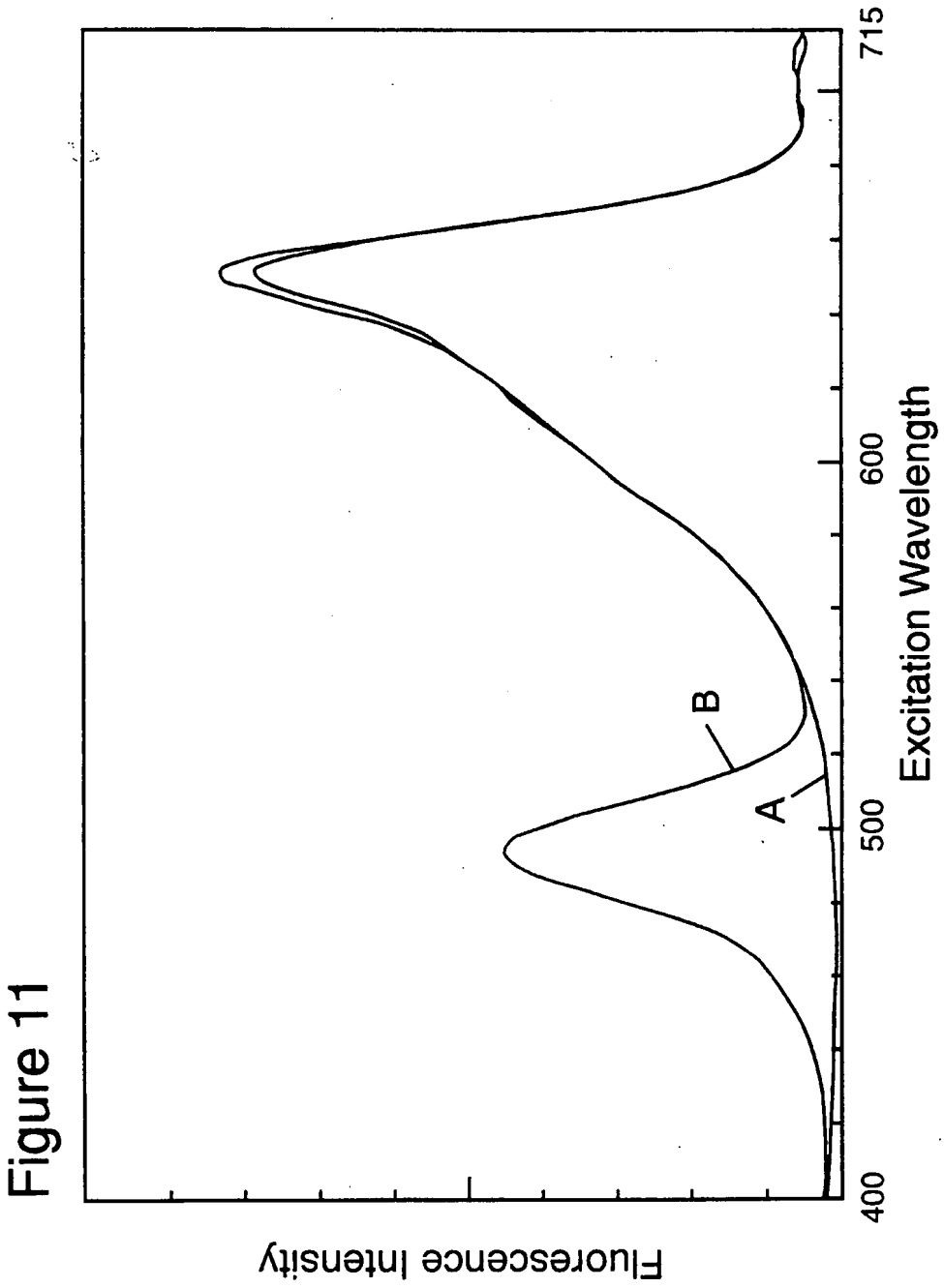

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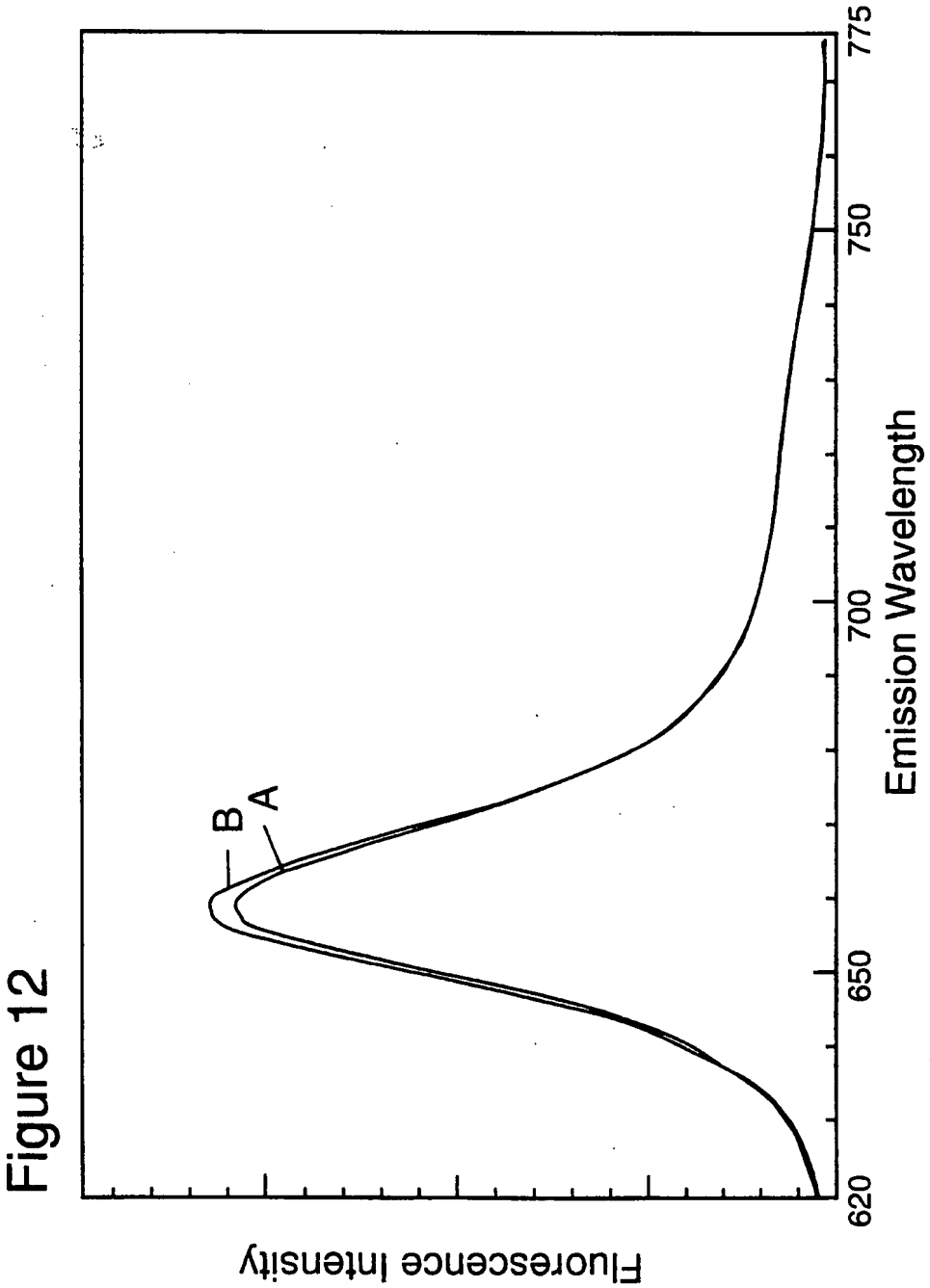

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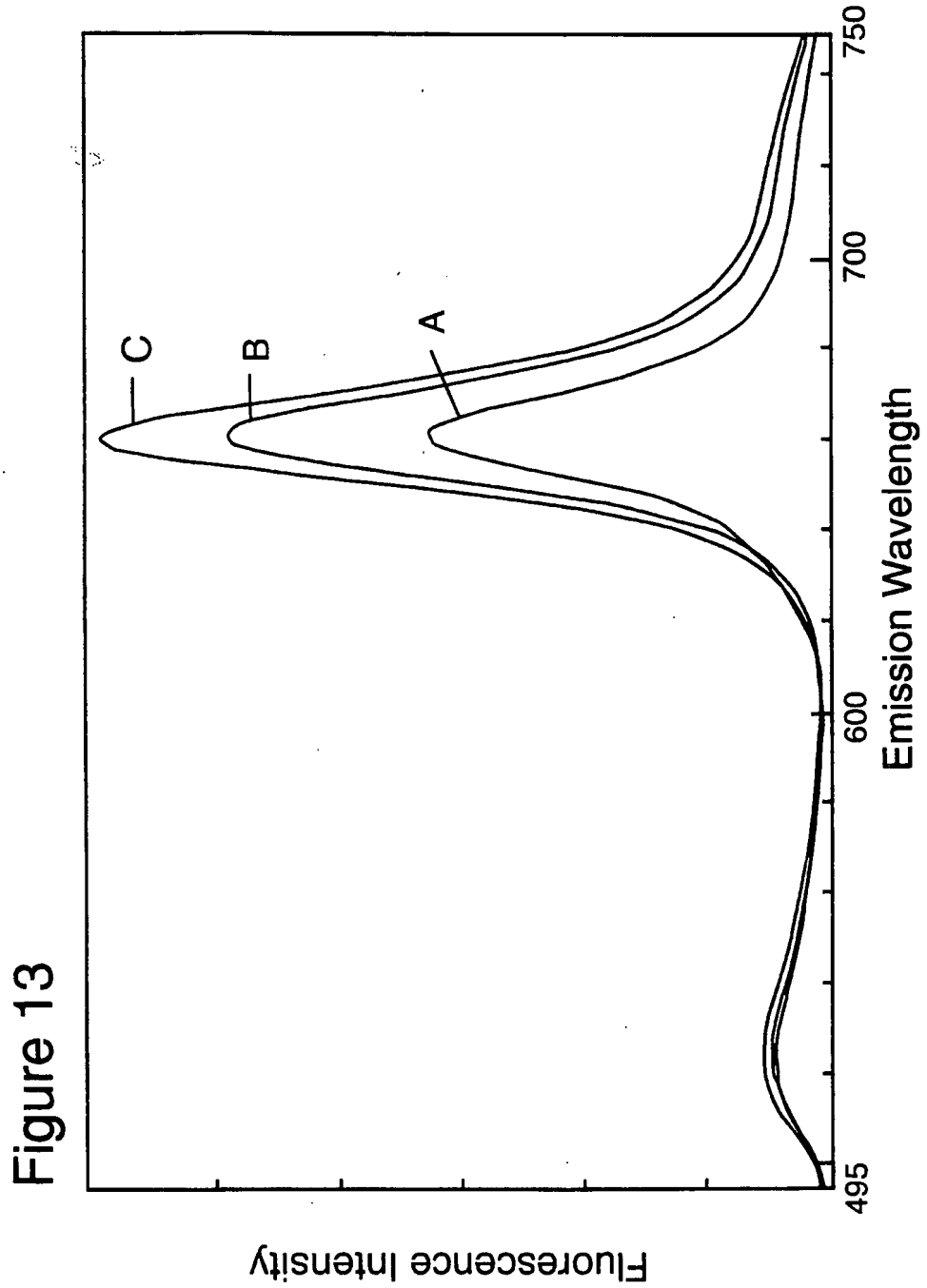
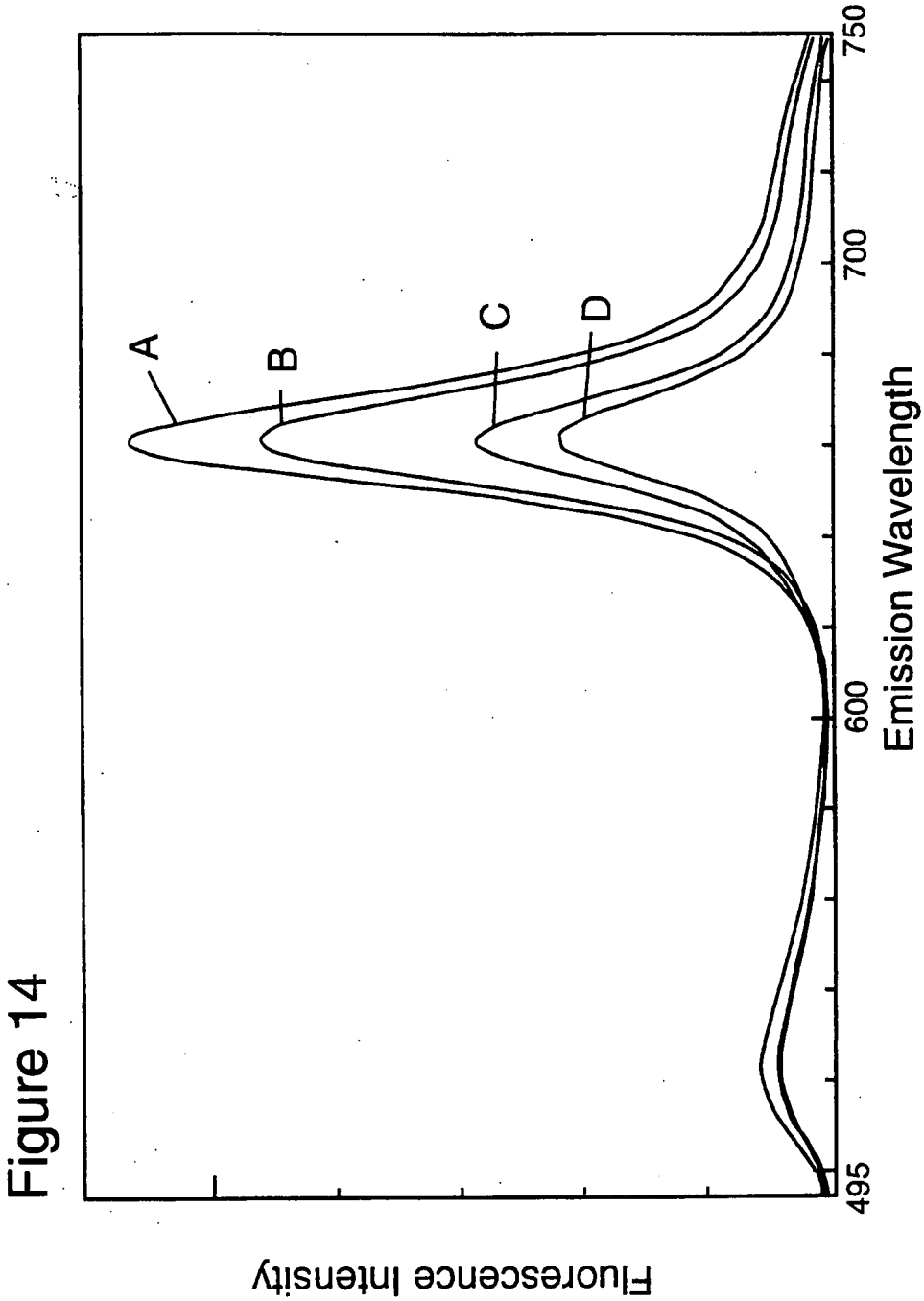


Figure 13



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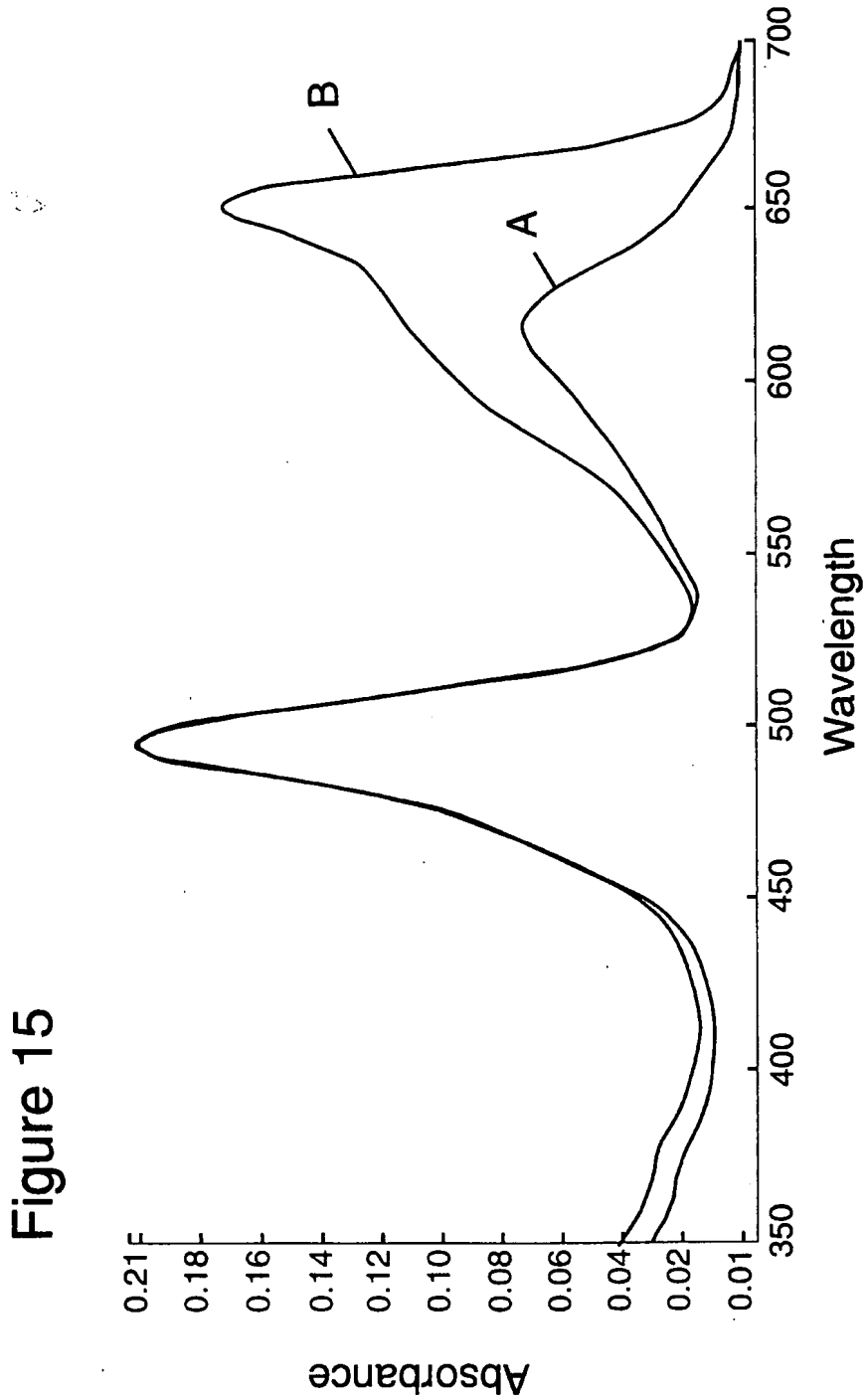

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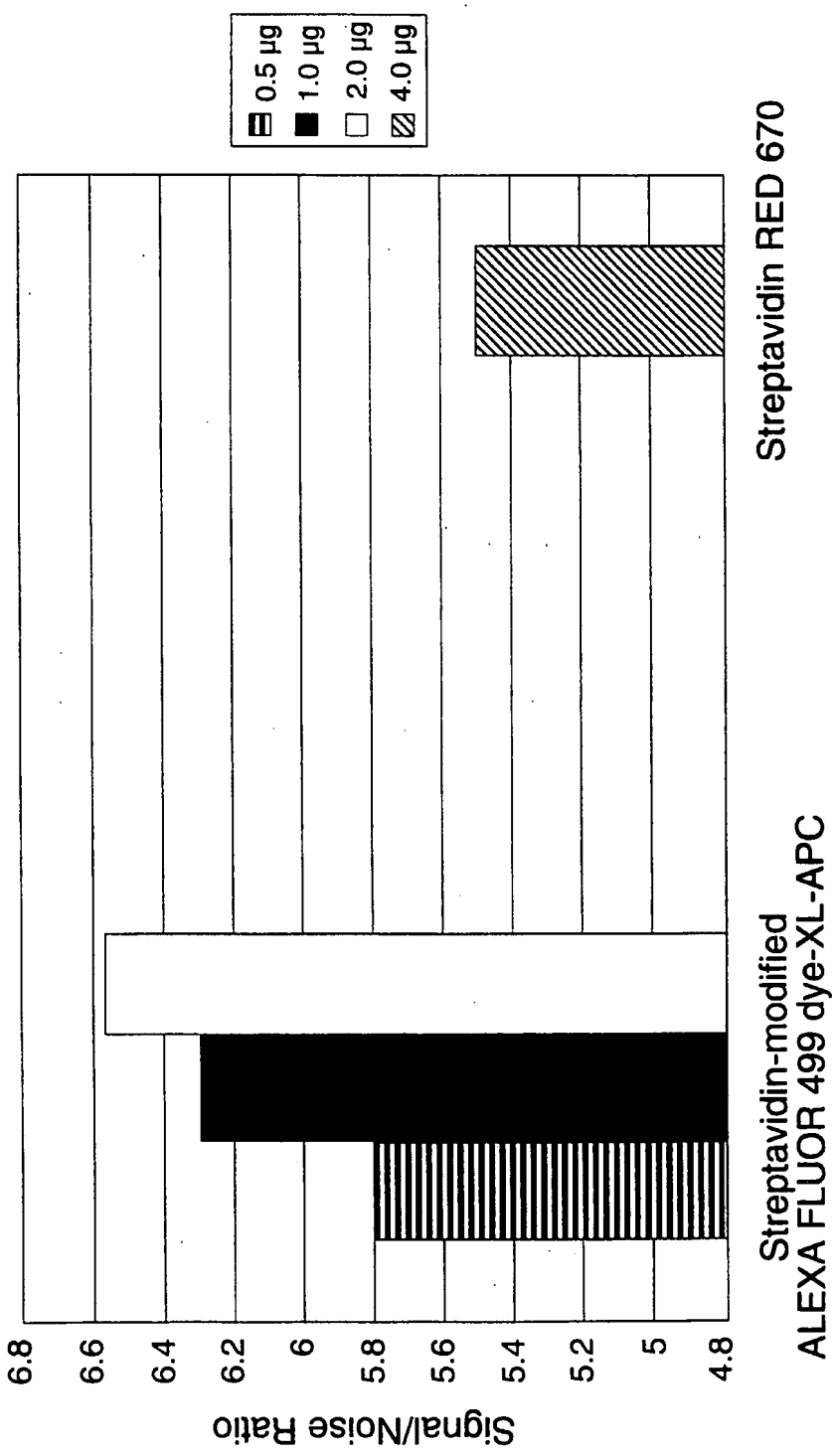
0.5 µg
1.0 µg
2.0 µg
4.0 µg
Streptavidin RED 670
Streptavidin-modified
ALEXA FLUOR 499 dye-XL-APC
Signal/Noise Ratio
6.8
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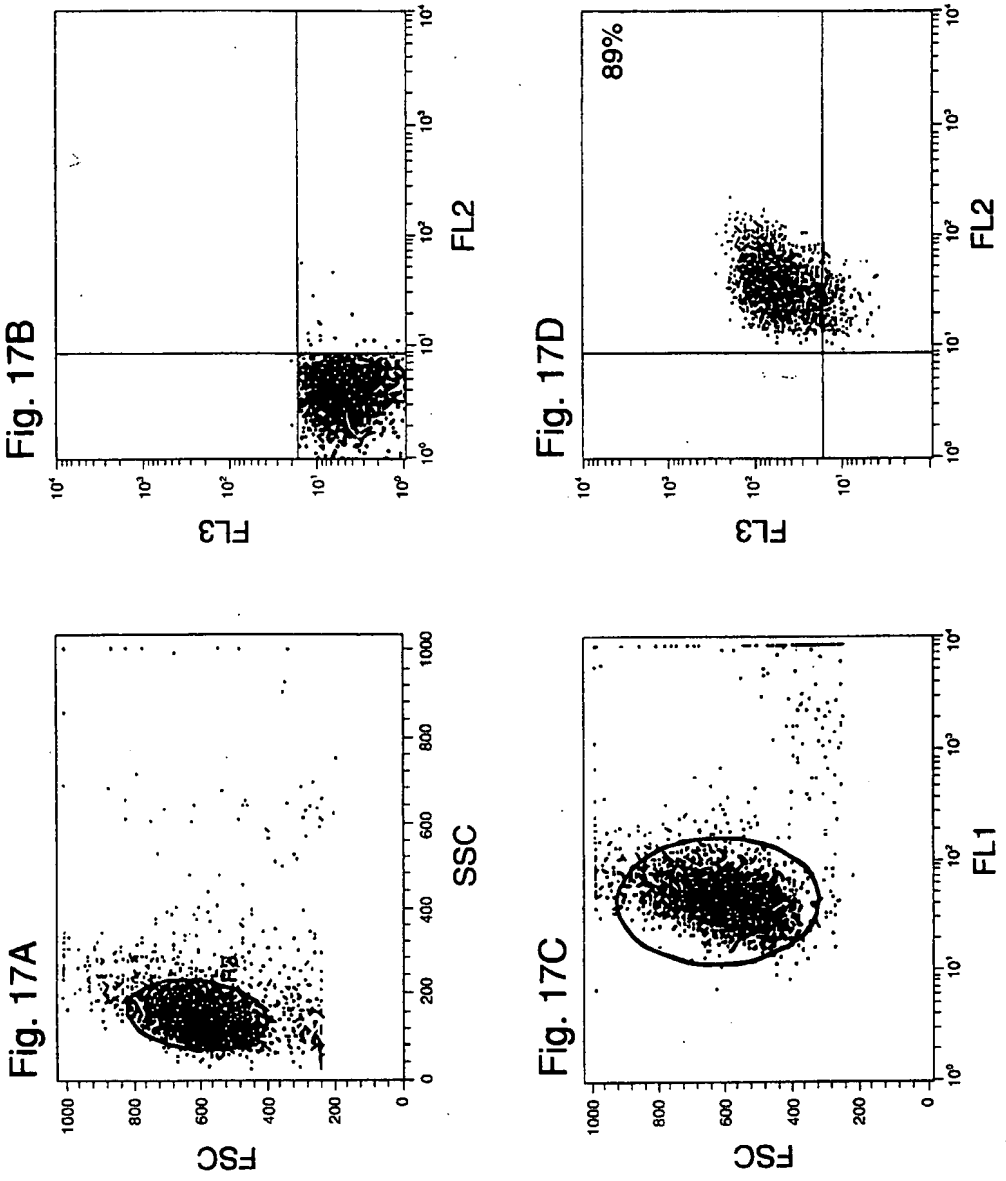
6.4
6.2
6
5.8
5.6
5.4
5.2
5
4.8

Figure 16

Figure 17



INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 99/22193

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/533 G01N33/58 G01N1/30 C07D311/82 C07K14/405

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C07D C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 714 386 A (ROEDERER MARIO) 3 February 1998 (1998-02-03) claims ---	1-10, 13-29
X	WO 97 39064 A (MOLECULAR PROBES INC) 23 October 1997 (1997-10-23) page 13, last paragraph page 44; table 10 ---	1-10, 13-15, 21-29
X	WO 94 05701 A (COULTER CORP) 17 March 1994 (1994-03-17) abstract; claims ---	1-10, 13-15, 21-29
X	US 4 666 862 A (CHAN JOSEPH L W) 19 May 1987 (1987-05-19) abstract --- -/--	1-10, 13-15

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 February 2000	16/02/2000

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Inte onal Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

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